Reconstitutions of plasmid partition systems and their mechanisms

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Abstract

Bacterial plasmid and chromosome segregation systems ensure that genetic material is efficiently transmitted to progeny cells. Cell-based studies have shed light on the dynamic nature and the molecular basis of plasmid partition systems. In vitro reconstitutions, on the other hand, have proved to be an invaluable tool for studying the minimal components required to elucidate the mechanism of DNA segregation. This allows us to gain insight into the biological and biophysical processes that enable bacterial cells to move and position DNA. Here, we review the reconstitutions of plasmid partition systems in cell-free reactions, and discuss recent work that has begun to challenge long standing models of DNA segregation in bacteria.

Introduction

In all forms of life, it is essential for DNA to be accurately segregated for the stable inheritance of genetic material. Eukaryotic cells use well-characterized mitotic spindles to segregate chromosomes via a tubulin-based mechanism. The processes that govern plasmids and chromosome segregation in bacterial cells are however, less well-understood. High copy-number plasmids rely on random diffusion to distribute replicated plasmids. Conversely, low-copy number plasmids and most chromosomes encode for dedicated partition (Par) systems to actively segregate DNA to daughter cells prior to cell division. The Par system encodes only two proteins, ParA and ParB, and a parS partition site. The parS site contains specific DNA sequences that act like a centromere, to which ParB binds to form the partition complex. ParA is an NTPase that binds and hydrolyzes ATP or GTP to provide the energy to drive DNA segregation. Three Par systems have been classified according to their respective NTPase: P loop ATPase with a deviant Walker A motif (Type I), actin-like ATPase (Type II) and tubulin-like GTPase (Type III) (reviewed in Gerdes et al., 2010; Baxter and Funnell,

2014). Although the loci that encode for Par systems have remarkable similarity in their genetic organization, fundamental differences in the sequence and structure of their NTPases have led to divergent plasmid partition mechanisms.

The advancement of fluorescence microscopy in the past few decades has transformed our view of bacterial subcellular organization. Bacteria are no longer seen as 'bags of enzymes', but instead to have highly organized structures. Bacterial cell biology has provided much insight into the subcellular organization of proteins, DNA and cellular compartments. Concomitantly, our knowledge of plasmid partition has progressed considerably with the aid of in vivo imaging. However, the knowledge that can be gleaned in vivo is limited by the resolution of the microscope and subcellular dynamics are convoluted by the complexities within the cell. Consequently, in vitro reconstitution is crucial for understanding the fundamental components that drive the cellular processes of a biological system. In vitro reconstitution uses a reductionist approach to create a minimal biological system and to identify the conditions required to reproduce in vivo dynamics. The plasmid partition system is a minimal system, consisting of only three components, hence making it an ideal model to reconstitute in a cell-free reaction. Table 1 indicates various in vitro reconstitutions of plasmid partition systems and the mechanisms derived from them. The technologies involved in cell-free reactions can be diverse and multifaceted, ranging from biochemical and molecular biology techniques used for the purification, labeling and reconstitution of components, to various fluorescence microscopy techniques for the imaging of system dynamics. In this review, we discuss the development of in vitro reconstitutions of plasmid partition systems and examine how they have advanced our understanding of the mechanisms underpinning bacterial DNA segregation.

Actin-like plasmid partition systems

Perhaps the most well-understood plasmid partition system is the E. coli R1 plasmid. The R1 plasmid encodes a type II ParMRC system that consists of an actin-like ATPase (ParM), an adaptor protein (ParR) and a partition site (parC) onto which ParR binds specifically. ParM is structurally similar to eukaryotic actin, forming two stranded filament bundles (Van den Ent et al., 2002). In vivo studies showed dynamic ParM filaments connecting plasmid pairs and forming elongated polymers, physically pushing the plasmids apart (Møller-Jensen et al., 2002). ParM polymerization only occurred in the presence of ParR and parC, prompting the idea that ParR/parC is responsible for stabilization of ParM filaments. These observations led to the hypothesis that dynamic polymerization of ParM provides the force to segregate plasmids. ParM undergoes dynamic switching between periods of growth and shrinkage, suggesting a "search and capture" mechanism where ParM filaments can continually explore the entire cell volume to bind a ParR/parC complex (Garner et al., 2004). This dynamic instability is a property that had previously only been observed in eukaryotic microtubules. Dynamic instability of ParM is driven by ATP hydrolysis and is crucial to plasmid partition. However, it remained unclear how ParM polymerization performs useful work to facilitate DNA transport.

The in vitro reconstitution of the three-component ParMRC system serves as an important step forward to understanding the mechanism (Garner et al., 2007). Beads were coated with Cy3-labeled parC DNA and mixed with ParR and Alexa488-labeled ParM. ParM filaments were observed to dynamically grow and shrink from the surface of the parC-beads. When dynamic filaments originating from different beads made contact, they stabilized to form a filament bundle. Continued elongation of the spindle pushed the beads apart, separating the beads over long distances (<120 μm), far exceeding the dimensions of a typical bacterial cell. Elongating ParM filaments were only observed between parC bead pairs,

suggesting that ParR/parC complexes bound to both filament ends and stabilized the unstable filaments, preventing their collapse. Conversely, unattached filaments exhibited dynamic instability and quickly depolymerized, allowing monomers to be recycled and relocate to polymerization sites on more stable filaments. Evidence for insertional polymerization was obtained through photobleaching experiments to show that polymerization occurs at the ends of the filament, near the plasmids (Møller-Jensen et al., 2003). Insertional polymerization was confirmed in vitro using photobleaching and speckle microscopy. Firstly, an elongating filament was photobleached and the intensity remained constant. Secondly, a sparse amount of Rhodamine-ParM was infused into the system for speckle microscopy. This showed direct incorporation of ParM monomers solely at the location of the partition complexes (Garner et al., 2007). The mechanism for spindle self-alignment was also investigated using micro-fabricated channels of various shapes (Campbell and Mullins, 2007). It was shown that the spindles aligned with the long axis of the channel. Elongation was seen to occur freely until the spindles encountered resistance at the poles or the bends of channels. For stabilized filaments to undergo elongation, a surplus of ParM monomers was required to add to the ends of the filament. This excess of monomers was provided by the collapse of the dynamically unstable, unbound ParM filaments, converting the free energy of unbound filaments into the elongating spindles. The indefinite growth of ParM filaments therefore ensures separation of plasmid pairs to opposite cell poles.

Tubulin-like plasmid partition systems

Type III tubulin-like Par systems consist of a GTPase (TubZ), an adapter protein (TubR), and a centromeric-site (tubC). The TubZRC system has been found to be encoded in numerous plasmids in the Bacillus genus, and poses a new form of plasmid partition system. TubZ from B. thuringiensis pBtoxis was found to assemble into dynamic linear polymers in vivo (Larsen

et al., 2007). These TubZ filaments are structurally similar to FtsZ/tubulin. Movement of TubZ filaments occurred via a treadmilling mechanism, where monomers assembled at the leading plus-end and disassembled at the trailing minus-end. This polarity contrasts with the bidirectional growth and collapse of ParM filaments. TubZ filaments did not exhibit dynamic instability, differentiating them from ParM polymers. Furthermore, ParM only formed filaments at physiological levels in the presence of ParR/parC, whereas TubZ polymerized even in the absence of TubR. The GTPase activity of TubZ was shown to be crucial to filament formation, with a TubZ mutant defective in GTP-hydrolysis assembling polymers at significantly lower levels than wild-type TubZ.

The pBtoxis TubZRC system from B. thuringiensis has only been recently reconstituted to explore their ability to transport DNA in vitro (Fink and Löwe, 2015). Atto488-labeled TubZ, TubR and Atto647-labeled tubC were mixed and imaged using TIRF microscopy. Dynamic growth and shrinkage of TubZ filaments and their interaction with TubR/tubC complexes was observed. Speckle microscopy was performed where Rhodamine-labeled TubZ incorporated solely at the plus-end of filaments and depolymerized from the minus-end. These experiments corroborated the TubZ treadmilling behavior previously observed in vivo. Binding of TubR/tubC to TubZ filaments resulted in a seven-fold decrease in depolymerization rate compared to unbound TubZ filaments. These results evidenced that TubR/tubC does not induce insertional polymerization, but instead reduces the rate at which subunits disassemble. It still remains to be seen exactly how cells use minus-end tracking for DNA segregation. Reconstitution of the TubZRC system suggests that a treadmilling TubZ filament exerts a pulling force on a TubR/tubC complex bound at the trailing end. This activity is consistent with in vivo observation of Bacillus cells, where TubZ filaments carrying DNA cargo travel along the long axis of a cell, depositing plasmids upon reaching the cell poles.

Walker-type plasmid partition systems

The type I Par system is the most widespread form of plasmid and chromosome segregation system across the bacterial kingdom. However, the mechanism of type I plasmid partition has been the most elusive. In the past decade or so, most reported partition systems have been based on a mitotic-like model in which 'cytoskeletal' ParA filaments push or pull plasmids apart. However, in the past few years, the diffusion ratchet model has emerged which instead focuses on dynamic ParA gradients as the driver of plasmid transport. Initial in vivo and in vitro observations supported filament-based models. In vivo studies identified diffuse clouds or helical structures of ParA that colocalized with the nucleoid and oscillated within the cell (Marston and Errington, 1999; Ebersbach and Gerdes, 2004; Fogel and Waldor , 2006., Hatano et al., 2007, Pratto et al., 2008). In vitro studies showed ParA forming filaments, suggesting that linear or helical ParA structures polymerize and depolymerize to position the partition loci (Barillà., 2005; Ebersbach et al., 2006; Ptacin et al., 2010). On the other hand, P1 ParA formed diffuse clouds over the nucleoid and discrete foci that blinked upon plasmid segregation (Hatano and Niki, 2010). In vivo observations of pB171 migrating behind ParA structures prompted a 'filament-pulling' model, in which extending ParA filament ends disassemble upon encountering ParB/parS complexes to pull plasmids towards the cell pole (Ringgaard et al., 2009). Extensive biochemical studies showed P1 ParA binding to nonspecific DNA in an ATP-dependent manner; and the slow conformational change of ParA, cycling between non-binding and DNA-binding states (Vecchiarelli et al., 2010). These data were inconsistent with ParA forming stable filaments required for force generation, but instead suggested the use of the nucleoid as a scaffold for plasmid motion. Therefore, a novel diffusion ratchet model was proposed where the time delay switch of ParA allows for it to diffuse and uniformly redistribute on the nucleoid. ParB loads onto the parS site, forming a high local concentration of ParB, and stimulating ATP hydrolysis of ParA on the partition

complex. The slow rebinding of ParA on the nucleoid, relative to the fast disassembly of ParA by ParB/parS creates an uneven distribution of ParA in the vicinity of the partition complex, driving plasmid motion.

A significant development on plasmid partition came with the in vitro reconstitutions of P1 and F plasmids (Hwang et al., 2013; Vecchiarelli et al., 2013). The cell-free reaction was performed using a flow cell coated with nonspecific DNA to form an immobilized DNA carpet that mimicked the bacterial nucleoid. Purified components consisting of ParA-GFP, ParB and Alexa647-labeled parS plasmids were mixed and infused into the flow cell and the system dynamics were visualized using a prism-based TIRF microscope. ParA-GFP coated the DNA carpet depending on ATP and ParA to ParB concentration ratios. Surprisingly, photobleaching experiments of ParA and ParB on the DNA carpet showed free protein exchange on the DNA carpet, contradicting earlier in vitro observations of stable ParA filaments. Partition complexes were observed to bind the DNA carpet for a short time before dissembling and dissociating from the flow cell surface. ParB-stimulated ATP hydrolysis accelerated ParA disassembly from the partition complexes and DNA carpet. This coupled with the time delay of ParA, resulted in depletion zones surrounding the partition complexes. The plasmid clusters displayed tethered Brownian motion that aided the formation of transient depletion zones by clearing the local vicinity of ParA. Eventually, the clusters dissociated and the depletion zone refilled. In the diffusion ratchet model, these depletion zones are thought to facilitate directed motion of partition complexes by inducing a local concentration gradient of ParA on the DNA carpet. However, this requires spatial confinement resembling the narrow gap between the nucleoid surface and the cell membrane in which plasmid partition is thought to occur. This was cleverly achieved by trapping parS-coated magnetic beads on the DNA carpet with an external magnetic field (Vecchiarelli et al., 2014). Under surface confinement, the trapped beads generated a persistent ParA depletion

zone on the DNA carpet by ParB-stimulated release. Strikingly, the beads displayed directed motion, chasing toward a higher ParA concentration gradient that propagated with the bead. Together, these reconstitutions support a diffusion ratchet model of plasmid motility in the absence of observable filamentous ParA. However, the dynamics of bidirectional segregation have yet to be reconstituted. Nevertheless, we would expect replicated plasmids to bidirectionally segregate as the merging of their depletion zones would drive them to move in opposite directions toward higher ParA concentrations.

Similar to the ParABS system, the MinCDE system self-organizes in E. coli to localize the cell division septum to midcell. MinD and ParA are both part of the family of P loop ATPases and both act as an ATP-dependent switch for binding to DNA (ParA) or cell membrane (MinD)(reviewed in Lutkenhaus 2012; Vecchiarelli et al 2012). An ensemble of in vitro reconstitutions of the Min system on planar membranes has contributed to the understanding of the similarities between MinD and ParA self-organization based on the reaction-diffusion mechanism (Loose et al., 2008; Ivanov and Mizuuchi 2010; Zieske and Schwille 2014; Vecchiarelli et al., 2016).

Recent progress on DNA segregation mechanisms

There have been recent developments on our understanding of DNA segregation mechanisms that have arisen from in vivo observations using super-resolution microscopy and in silico modeling. The in vitro reconstitutions of P1 and F plasmid dynamics have provided much mechanistic insight into plasmid partition. However, the debate is still evolving as to how the Par system transports and localizes partition complexes in the cellular environment. In C. crescentus, 3D super-resolution microscopy showed that the directed movement of chromosomes is the result of ParB/parS chasing the trailing edge of a ParA gradient across the nucleoid (Ptacin et al., 2014). Computer modeling suggested that diffusion-binding of

partition complexes is insufficient for ParA-mediated DNA transport and proposed a 'DNA-relay' model, where the partition complex utilizes ParA-DNA tethers and the elastic properties of chromosomes to translocate across the nucleoid (Lim et al., 2014; Surovtsev et al., 2016). An alternative model showed plasmid transport as a Brownian ratchet, mediated by the forces of ParA-ParB interactions (Hu et al., 2015; Jindal and Emberly, 2015). The mechanochemical model demonstrated that collective binding and dissociation of ParA-ParB bonds are able to tether the plasmid and quench random diffusion, providing for the directed motion along a ParA gradient (Hu et al., 2015).

A recent notable finding revealed that partition complexes from F plasmid and B. subtilis chromosome are located within the nucleoid interior and colocalized with dense chromosome regions (Le Gall et al., 2016). Similar to C. crescentus, ParA filaments were found to be absent. Hence, an adapted diffusion ratchet named the 'hitch-hiking' model was proposed, in which ParA localize with dense DNA regions within the nucleoid. Partition complexes are then transported between these dense chromosome regions driven by local ParA gradients. This is further supported by super-resolution images of TP228 ParF forming a 3D polymeric meshwork that oscillates within the nucleoid for plasmid transport (McLeod et al., 2016). It was proposed that the meshwork acts as a 'Venus flytrap' that captures ParG-plasmids via ParF-ParG interactions. ParG stimulates ParF disassembly, creating a less dense mesh at the trailing edge that releases the ParG-plasmids. The dynamic remodeling of ParF mesh by ParG generates an oscillating gradient of meshwork in the cell to continuously capture and release the plasmids to fine tune their positions.

Previously, the diffusion ratchet mechanism was based on the postulation that the nucleoid takes up a sizeable volume of the bacterial cell and that large plasmids would be excluded from the nucleoid (reviewed in Vecchiarelli et al., 2012). Hence, the plasmids would exploit the nucleoid and cell membrane interface to traffic along the surface-mediated

ParA gradient. Given the latest findings, this would imply that partition complexes are instead caged within the nucleoid interior, providing a 3D confinement for the partition complexes to move along a volume-mediated ParA gradient or meshwork. The spatial organization of the ParA gradient would be dependent on the underlying structure of the nucleoid scaffold, as well as the dynamics of nucleoid macromolecular crowding. The spatiotemporal dynamics of the partition system in relation to the nucleoid structure remains to be explored using super-resolution microscopy.

Super-resolution microscopy has initiated a major shift in how we view plasmid and chromosome segregation in bacteria, from ParA filament-based models toward gradient-based mechanisms, involving patches or meshwork of ParA dimers or oligomers binding to the nucleoid. Although these techniques have proved to further our understanding of DNA segregation, it is important to recognize that they can be prone to artifacts. Many of these artifacts can be attributed to the use of unsuitable fluorescent-fusion proteins (Landgraf et al., 2012; Swulius and Jensen, 2012). It is therefore important that super-resolution microscopy be used as a complementary tool to other established techniques.

Summary

In vitro reconstitution is an important method for investigating minimal systems in the absence of any extraneous cellular components. Through the reconstitution of biological systems required for basic cellular processes, we can better understand how these processes work on a molecular level. In this review, we have shown how in vitro reconstitution, in combination with in vivo cell imaging and super-resolution microscopy, has allowed for a deeper understanding of the diverse structures and dynamic processes which contribute to spatial organization of DNA within bacterial cells. The actin-like partition system is now fairly well-characterized and extensive knowledge of ParM filament self-assembly and

structure has been gained through methods such as cryo-electron microscopy (Bharat et al., 2015). Bidirectional segregation of plasmid cargo has yet to be achieved using TubZRC and further reconstitutions are required to replicate the plasmid dynamics observed in vivo. Additionally, reconstituting the dynamics of treadmilling TubZ filaments within a confined geometry could also provide insight into how plasmids are deposited at cell poles. For Walker-type partition system, the in vitro reconstitutions of plasmid transport by ParA gradient, the lack of ParA filaments in vivo and the localization of partition complexes within the nucleoid, together suggest that Par-mediated chromosome segregation could also be driven by Brownian ratchet-type mechanism. An important next step is to reconstitute the chromosomal Par system. From here we would be able to gain a clearer understanding of the role of each Par component and the nucleoid in chromosome segregation, and reveal whether partition complex dynamics self-organize as a minimal system, or as part of a larger, more complex system.

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Conflict of interest

The authors declare no conflict of interest.

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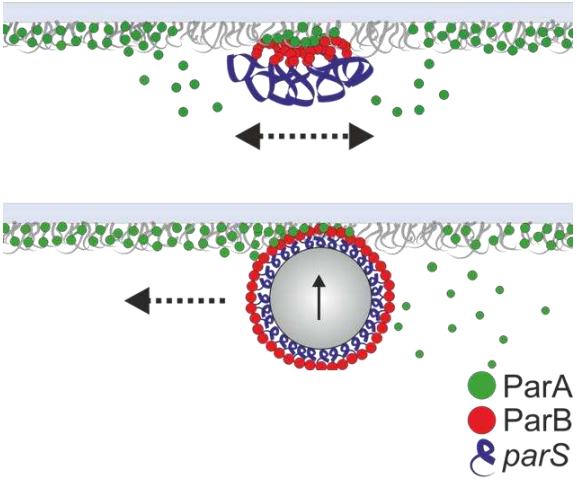
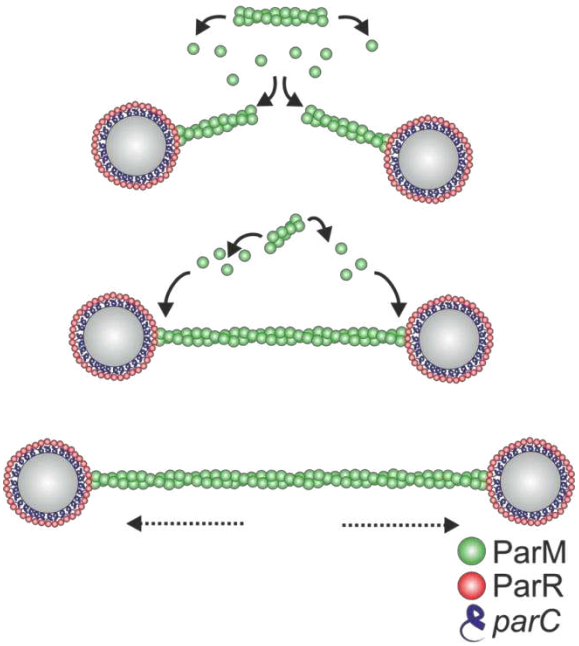
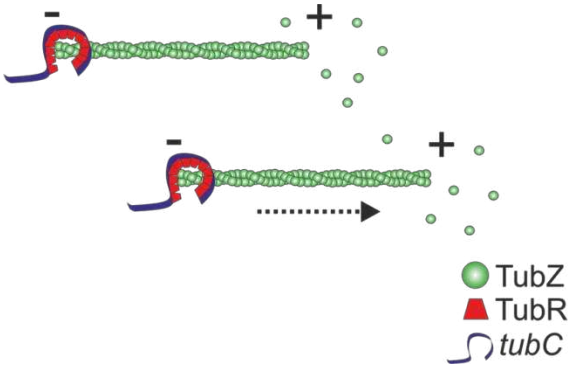
427 **Table 1: Reconstitutions of plasmid partition systems and their mechanisms**

Type	Plasmid/ Partition System	Host	In vitro reconstitution*	Mechanism	References
I	P1 ParABS F SopABC	E. coli		Diffusion ratchet mechanism	Hwang et al. 2013, Vechiarelli et al. 2013, 14
II	R1 ParMRC	E. coli		Insertional polymerization mechanism	Garner et al. 2007
III	pBtoxis TubZRC	B. thuringiensis		Treadmilling mechanism	Fink et al. 2015

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