Nonspecific DNA binding by P1 ParA determines the distribution of plasmid partition and repressor activities

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The faithful segregation, or “partition,” of many low-copy number bacterial plasmids is driven by plasmid-encoded ATPases that are represented by the P1 plasmid ParA protein. ParA binds to the bacterial nucleoid via an ATP-dependent nonspecific DNA (nsDNA)-binding activity, which is essential for partition. ParA also has a site-specific DNA-binding activity to the par operator (parOP), which requires either ATP or ADP, and which is essential for it to act as a transcriptional repressor but is dispensable for partition. Here we examine how DNA binding by ParA contributes to the relative distribution of its plasmid partition and repressor activities, using a ParA with an alanine substitution at Arg351, a residue previously predicted to participate in site-specific DNA binding. In vivo, the parAR351A allele is compromised for partition, but its repressor activity is dramatically improved so that it behaves as a “super-repressor.” In vitro, ParAR351A binds and hydrolyzes ATP, and undergoes a specific conformational change required for nsDNA binding, but its nsDNA-binding activity is significantly damaged. This defect in turn significantly reduces the assembly and stability of partition complexes formed by the interaction of ParA with ParB, the centromere-binding protein, and DNA. In contrast, the R351A change shows only a mild defect in site-specific DNA binding. We conclude that the partition defect is due to altered nsDNA binding kinetics and affinity for the bacterial chromosome. Furthermore, the super-repressor phenotype is explained by an increased pool of non-nucleoid bound ParA that is competent to bind parOP and repress transcription.

The partition of low-copy prokaryotic plasmids typically requires a pair of proteins: a sequence-specific DNA-binding protein that recognizes the DNA partition site (the prokaryotic centromere) and an ATPase or GTPase that serves to mobilize and localize the plasmid in the cell (reviewed in Ref. 1). These Par systems are broadly divided into three classes, characterized by their NTPase protein and mechanism of action: Walker-type ATPases, actin-like ATPases, and tubulin-like GTPases. The Escherichia coli P1 plasmid is representative of the Walker-type ATPase family, encoding the ATPase protein ParA. The site-specific DNA-binding protein, ParB, binds to the partition site parS. A subgroup of ATPases within the Walker family, which includes P1 ParA, are also transcriptional repressors. P1 ParA acts to repress the par operon through specific binding to the parOP operon sequence (2, 3). This repression by ParA is strongly stimulated by ParB, and together they autoregulate transcription of the par operon.

The Walker class of partition ATPases is the most prevalent class identified among plasmid genomes. Many bacterial genomes also contain parA- and parB-like genes, of which the ParAs are similarly of the Walker-type class. These ParAs are evolutionarily related to the MinD ATPases that drive localization of the cell division machinery, and together they represent a larger family of cellular positioning proteins (4–7). These ATPases pattern themselves on various scaffolds in vivo (the bacterial nucleoid for ParA; the membrane for MinD) with the assistance of their cognate partner proteins (ParB and MinE, respectively). During plasmid partition, ParB-parS complexes interact with nucleoid-bound ParA, promoting redistribution of ParA on the nucleoid (8–17). The ability of ParB-parS to stimulate the interconversion of ParA between nucleoid-bound and nucleoid-free states, and to follow a moving gradient of nucleoid-bound ParA, led us to the conclusion that plasmid movement arises through a diffusion-ratchet version of a Brownian ratchet mechanism (reviewed in Ref. 18).

Binding and hydrolysis of ATP serve multiple roles in P1 ParA function. ParA possesses a weak intrinsic ATPase activity that is stimulated by ParB and DNA, and ATP binding and hydrolysis are required for partition (19–21). Two different modes of DNA binding by ParA are driven through binding of adenine nucleotides, and DNA sequence specificity differs according to the nucleotide bound. ADP is preferred over ATP for its site-specific binding activity to parOP (22). Nonspecific DNA (nsDNA) binding by ParA, however, is absolutely dependent upon ATP (9, 12, 17, 23, 24). Upon binding ATP, P1 ParA undergoes a slow conformational change to [ParA-ATP]*, which confers upon it the ability to bind nsDNA (12). The slow rate at which [ParA-ATP]* forms is necessary to establish the gradient of ParA on the nucleoid through interactions with ParB. The dynamic formation and redistribution of ParA gradients and of plasmid-ParB complexes on nsDNA has been recapitulated in a cell-free system by total internal reflection fluorescence (TIRF) microscopy (13, 14). Biochemically, a large complex of P1 ParA, ParB, and DNA can be isolated and observed to assemble and disassemble in the presence of ATP (25). The complex is called NAC (nucleoid adaptor complex) because its properties are representative of the ParA-ParB actions that drive the transient plasmid-nucleoid interactions of plasmid partition in vivo.

Mutation of the catalytic lysine residue (Lys122) has allowed us to dissect the contributions of the various ParA-ADP/ATP
were involved in its site-specific DNA-binding activity (27). A K122Q mutation in the catalytic lysine blocks the conformational change from ParA-ATP to [ParA-ATP]* and prevents nsDNA binding but not site-specific binding to parOP. In vivo, the change destroys its partition activity, but creates a “super-repressor,” which bypasses the corepressor activity of ParB; that is, its repressor activity is high and equivalent to that of WT ParA in the presence of ParB. These observations suggested that ParA-ADP and ParA-ATP, but not [ParA-ATP]*, represent the repressor conformation of ParA, and furthermore, that ParB acts as a corepressor by converting [ParA-ATP]* back to ParA-ADP/ATP.

Discrete domains of ParA define its role in repression and partition. The majority of the protein core sequence defines the Walker ATPase domain (27, 28). The subgroup of ATPases to which P1 belongs possesses additional N-terminal sequence that contains the transcriptional repression function. In domain-swapping experiments, the N-terminal 66 residues of ParA were found to be necessary for binding to parOP, acting as a specificity factor that discriminates between P1 parOP and that of the homologous P7 plasmid system (29). Crystallographic data later confirmed that the N-terminal specificity region contained an HTH motif that was part of a larger winged HTH (winged helix-turn-helix) domain (27). A model of P1 ParA-ADP bound to parOP DNA generated from the crystal structure suggested that two distinct regions of the protein were involved in its site-specific DNA-binding activity (27). These included the wHTH domain as previously predicted as between the specific DNA sequences that are bound by the N-terminal wHTH domains of ParA. Indeed, ParA R351A was impaired in its DNA binding, and how those activities participate in repression and partition. To assess partition function but its repressor function is greatly enhanced and that it behaves as a super-repressor. Biochemical dissection indicated that both phenotypes can be explained by altered affinity and kinetics for interacting with nsDNA. Together, the data illustrate the importance of R351A in nsDNA binding by P1 ParA, and importantly, the switching role of ParA in binding different DNA substrates in both partition and repression of the P1 partition operon.

**Results**

**ParA**<sup>R351A</sup> is a super-repressor with impaired partition function in vivo

The crystal structure of P1 ParA was previously modeled bound as a dimer to DNA at parOP, which predicted that the Arg<sup>351</sup> residue may play a role in ParA DNA binding to parOP and therefore in repression (27). In the model, the Arg<sup>351</sup> residues of each monomer are positioned close to the DNA between the specific DNA sequences that are bound by the N-terminal wHTH domains of ParA. Indeed, ParA<sup>R351A</sup> was slightly impaired for binding to the parOP sequence in vitro (27). However, mutation of the analogous residue in F SopA indicated a defect in its partition and not repressor activities (23), so we initiated an analysis of ParA<sup>R351A</sup> to clarify the role of this residue in repression and partition. To assess partition function in vivo, we monitored the stability of a miniP1 <sup>D</sup>parAB plasmid that contains parS but lacks parA and parB (pBEF246, Table 1). We measured the retention of miniP1<sup>D</sup>parAB and parB supplied in trans from a pBR322-derived plasmid (21). In the presence of WT parA and parB (pEF5, Table 1), 90 ± 4% of E. coli cells retained miniP1<sup>D</sup>parAB, compared with 14 ± 7% miniP1 retention without parA and parB (the vector pBR322). In the presence of parA<sup>R351A</sup> and parB<sup>+</sup> (pBEF356, Table 1), 43 ± 6% of cells retained miniP1<sup>D</sup>parAB. Therefore, plasmid maintenance promoted by parA<sup>R351A</sup> is compromised although not completely defective.

We next monitored repression by assessing the ability of parA to repress lacZ expression under the control of the parOP operator and promoter sequence (21). We used both a single-copy and multicopy reporter systems, which afforded better discrimination of the high and low levels of β-gal produced. WT ParA is a weak repressor alone, but very strong in the presence of the co-repressor ParB (2) (Fig. 1). In contrast to our original prediction, we found that ParA<sup>R351A</sup> was a proficient repressor, and that its repressor activity was largely insensitive to parB. In the presence of parB, repression by parA<sup>WT</sup> and parA<sup>R351A</sup> were similar and so strong that β-gal levels were measurable only with the high-copy reporter (Fig. 1B). In fact,
parB stimulated the repressor activity of parA R351A only slightly (less than 2-fold, compared with over 50-fold for parA WT; Fig. 1). Therefore, the repressor activity of parA R351A effectively bypassed the need for the corepressor activity of parB, and placed the R351A allele into the class of ParA that we call super-repressors.

The in vivo results indicated that the R351A change partially compromised partition activity but stimulated the repressor activity of ParA. With this in mind, we proceeded to biochemically dissect the functions of ParAR351A to answer two questions: first, how does the R351A change damage P1 partition activity, and second, how does the change bypass the need for the co-repressor ParB and create a super-repressor?

**ATPase activity and [ParA-ATP]* formation are unaltered by the R351A mutation**

To characterize the defect(s) responsible for the in vivo phenotype of ParA R351A, we first assessed its response to ATP binding by examining its ATPase activity and its ability to undergo the conformational change to form [ParA R351A-ATP]*, both of which are required for partition activity (Fig. 2) (12, 20). We saw no appreciable difference in ATPase activity between ParA WT and ParA R351A, with or without ParB (Fig. 2A). ParB was able to stimulate the ATPase activity of both proteins to similar extents. Therefore, the R351A change did not damage either the ATPase activity of ParA or the interaction with ParB that is necessary for stimulation of this activity.

The transition from ParA-ATP to [ParA-ATP]* has been characterized through monitoring the intrinsic fluorescence of a singular tryptophan (Trp126) in ParA, which is slowly reduced in response to ATP binding (12). Using this approach, we found no significant difference between ParA WT and ParA R351A upon the addition of ATP (Fig. 2B), indicating that ParA R351A was not impaired for adopting the [ParA-ATP]* form.
PARA-DNA interactions during partition and expression

Figure 3. NAC formation by ParA WT and ParAR351A. NAC formation by ParA WT (blue) and ParAR351A (red) were monitored by 90° light scattering as described previously (25). ParA and ParB were incubated at the indicated concentrations in the presence of 50 μg/ml of nsDNA for 15 min at 23 °C. The sample was briefly removed from the spectrofluorimeter (−5 s, gray vertical bar) to add ATP (to 0.5 mM), and returned to the instrument to follow scattered light in real time. Traces shown are the average of 4 replicates each, with the S.D. of the traces indicated in the lighter hue.

ParAR351A forms smaller, less stable NACs

The next step in the partition complex assembly that we can measure in vitro is the formation of the large NAC complexes, which requires ParA, ParB, DNA, and ATP, and can be detected by a 90° light scattering assay in real time (25). ParA, ParB, and DNA are mixed and allowed to equilibrate, and then the reaction is initiated by the addition of ATP. Following a lag, the signal increases as the complex assembles and then decreases to a plateau representing disassembly (Fig. 3). The lag reflects the slow [ParA-ATP] to [ParA-ATP]* transition but also depends on the relative concentrations of ParA, ParB, and DNA (25). Using this assay, we observed that the ability of ParAR351A to assemble NACs was significantly reduced compared with that of ParA WT, and the complexes that did form were less stable. First, under conditions in which WT ParA forms robust complexes (2 μM ParA and 1 μM ParB), ParAR351A was unable to form any discernable NAC complex (Fig. 3). Raising the concentration of ParB (2 μM) or ParA and ParB (4 and 2 μM, respectively) promoted some complex assembly by ParAR351A, but to a smaller extent and with lower stability compared with WT complexes (Fig. 3). These data show that ParAR351A is able to form NACs, but the assembly and stability of these complexes are significantly compromised.

nsDNA binding by ParAR351A is more labile than that by ParA WT

The ATP-dependent nsDNA-binding activity of Walker class ParA ATPases is necessary for partition and is a crucial property of the Brownian-ratchet models of plasmid partition that have been proposed (12, 15–17, 23, 24, 30, 34). The requirement for ATP and DNA, and the slow lag during P1 NAC assembly indicate that the [ParA-ATP]* form is necessary and predicts that the nsDNA-binding activity of ParA is also required for complex assembly. Because ParAR351A can form [ParA-ATP] (Fig. 2B), we next asked whether it had a defect in nsDNA binding that could explain the partition/NAC assembly defects. As the substrate, we used fluorescent Alexa 514-labeled nsDNA substrate that contains Alexa 514 moieties evenly distributed on the DNA, and whose fluorescence is quenched by ParA binding (12). In this assay, ParA and DNA were mixed, transferred to a fluorimeter cuvette, and baseline fluorescence was measured for ~30 s (Fig. 4A). DNA binding was initiated by the addition of ATP (the cuvette was briefly removed from the fluorimeter for ATP addition and rapid mixing (~5 s)), and then monitored in real time. Using this approach, we did not see any significant difference in nsDNA binding between WT and R351A proteins (Fig. 4A). Because NAC complexes were less stable with ParAR351A than with ParA WT, we then examined the off rate of both ParA proteins from the DNA substrate following the addition of excess unlabeled competitor nsDNA after binding saturation had occurred. Under these conditions, ParAR351A dissociated from the DNA substrate much more rapidly than the WT protein dissociated from DNA (Fig. 4A). WT ParA protein equilibrated with the added competitor nsDNA ~70 s (k d = 4.8 × 10−2 ± 5.0 × 10−4 s−1) after its addition, whereas ParAR351A equilibrated with the added competitor faster than we could measure using our fluorimeter (~5 s). Although the extent of nsDNA binding by ParAR351A did not appear significantly different from that of ParA WT in this assay, the rates with which they dissociated were substantially different.

To measure the association and dissociation kinetics without the 5-s interruption necessary for experiments in our spectrofluorimeter, we employed biolayer interferometry (BLI) to examine the ParA-DNA interaction. BLI detects analyte (protein) interactions with a ligand (DNA) that is conjugated to the surface of a probe (biosensor). BLI measures changes in optical thickness of the sensor surface by measuring and comparing the interference pattern from light reflected by the sensor surface with and without protein binding (35–37). We utilized ForteBio’s Octet™ RED96, which allowed us to examine up to
ParA-DNA interactions during partition and expression

Figure 4. nsDNA-binding activity of ParA. The DNA binding capability of ParA proteins was measured by (A) monitoring fluorescence of Alexa 514-labeled nsDNA and the baseline fluorescence signal was monitored for 30 s at room temperature. DNA binding was initiated by the addition of ATP (gray vertical bar represents the −5 s the sample was out of the spectrophotometer) and fluorescence was further monitored for 300 s. The sample was again removed briefly from the spectrophotometer to add unlabeled competitor nsDNA (sonicated salmon sperm DNA at the indicated mass ratios). Fluorescence counts were normalized to the average signal prior to the addition of ATP, and adjusted for dilution effects at each addition. Each addition of competitor nsDNA was conducted in duplicate, with the average of both traces indicated. Pre- and post-ATP traces are the average of all mass ratios). Fluorescence counts were normalized to the average signal prior to the addition of ATP, and adjusted for dilution effects at each addition. Each addition of competitor nsDNA was conducted in duplicate, with the average of both traces indicated. Pre- and post-ATP traces are the average of all mass ratios). Fluorescence counts were normalized to the average signal prior to the addition of ATP, and adjusted for dilution effects at each addition. Each addition of competitor nsDNA was conducted in duplicate, with the average of both traces indicated. Pre- and post-ATP traces are the average of all mass ratios). Fluorescence counts were normalized to the average signal prior to the addition of ATP, and adjusted for dilution effects at each addition. Each addition of competitor nsDNA was conducted in duplicate, with the average of both traces indicated. Pre- and post-ATP traces are the average of all mass ratios). Fluorescence counts were normalized to the average signal prior to the addition of ATP, and adjusted for dilution effects at each addition. Each addition of competitor nsDNA was conducted in duplicate, with the average of both traces indicated. Pre- and post-ATP traces are the average of all mass ratios). Fluorescence counts were normalized to the average signal prior to the addition of ATP, and adjusted for dilution effects at each addition. Each addition of competitor nsDNA was conducted in duplicate, with the average of both traces indicated. Pre- and post-ATP traces are the average of all mass ratios). Fluorescence counts were normalized to the average signal prior to the addition of ATP, and adjusted for dilution effects at each addition. Each addition of competitor nsDNA was conducted in duplicate, with the average of both traces indicated. Pre- and post-ATP traces are the average of all mass ratios). Fluorescence counts were normalized to the average signal prior to the addition of ATP, and adjusted for dilution effects at each addition.

Biases in affinity of [ParAR351A-ATP]* for both nsDNA and parOP drive super-repression

Our previous work suggested that ParA-ADP and ParA-ATP, but not [ParA-ATP]*, represented the repressor conformation of ParA (12, 21, 26, 38). First, the ADP form of ParA bound better than the ATP form to parOP in vitro. Second, ParA mutants, such as ParA K126Q, which could not undergo the ParA-ATP to [ParA-ATP]* conformational change, were super-repressors; that is, they were locked into the repressor form and thus bypassed the need for ParB corepressor activity. The observation here that ParAR351A, which can form [ParA-ATP]*, is also a super-repressor made us consider the possibility that [ParA-ATP]* does bind site-specifically to parOP, but this activity is normally out-competed by nsDNA, in which case ParA-ADP would appear to bind better than [ParA-ATP]* to parOP because the former cannot bind nsDNA.

We are able to separate the repression and partition functions of ParA and ParB in vitro and in vivo using our assays. However, in the bacterial cell these activities are not discretely compartmentalized and occur concurrently. A cellular abundance of ATP likely drives the majority of ParA in the cell into ParA-ATP*, whereas a smaller subset exists in the ParA-ADP/ATP state. Between the distributions of these states in vivo, repression at parOP must take place. Because of this, we speculated that because [ParAR351A-ATP]* binds to nsDNA in a reduced capacity (Fig. 4B), a significant fraction of the [ParAR351A-ATP]* pool in the bacterial cell is dissociated from the nucleoid and available to bind to parOP. If the [ParA-ATP]* state is capable of binding to parOP, a change in distribution pools should make [ParAR351A-ATP]* a better repressor than [ParA-ATP]* in the absence of ParB. In other words, we considered the possibility that the relative competition between the site-specific and nonspecific DNA-binding activities of ParA was altered by the R351A mutation, in favor of site-specific DNA binding so ParAR351A behaved as a super-repressor.
To test this hypothesis, we designed two new biotinylated DNA substrates: parOPBT, a 348-bp singly biotinylated fragment containing parOP, and parOpdelBT, a 353-bp singly biotinylated fragment that mimicked nsDNA by truncating one of the imperfect repeats, damaging the parOP sequence for ParA binding, but maintaining a similar composition to parOPBT (3). We then employed BLI to examine the binding of ParA to both parOPBT and parOpdelBT, and the relative levels of competition by added nsDNA.

First, we confirmed that DNA binding by ParA, when bound to ADP, was specific for parOP in this assay (Fig. 5A). As predicted, both ParA-ADP and ParA351A-ADP bound only to parOPBT (solid lines) and not to parOpdelBT (dashed lines) (Fig. 5A). Similar to previous experiments (27), we see that ParA351A was only modestly reduced for binding to parOP. The dissociation rate of ParA351A (3.3 × 10⁻² ± 7.7 × 10⁻⁵ s⁻¹) was also only modestly increased with respect to that of ParAWT (2.1 × 10⁻² ± 3.9 × 10⁻⁵ s⁻¹).

We next compared the binding extents of both parOPBT and parOpdelBT substrates in the presence of ATP and increasing concentrations of competitor nsDNA. As the parOP site (~40-bp) is within the context of the larger nsDNA fragment (~350-bp), we needed to discriminate between binding at the specific (parOP) and nonspecific sequence regions. We rationalized that as we increased the amount of competitor nsDNA present during the association of ParA-ATP with the DNA bound to the biosensor, we should incrementally compete away binding to the nonspecific regions, elucidating relative differences in parOP and nsDNA binding between ParAWT and ParA351A.

In the presence of ATP but the absence of added competitor nsDNA, the inclusion of parOP on a DNA fragment reduced ParAWT and increased ParA351A binding (Fig. 5B, upper traces in each panel), illustrating that the relative affinities for specific and nonspecific DNA differed between ParAWT and ParA351A. The addition of competitor nsDNA to the solution reduced the binding by both ParAWT and ParA351A to parOP DNA. Importantly, WT ParA binding to parOP was more sensitive than ParA351A to competition by nsDNA (Fig. 5, B and C, right panel). This sensitivity is illustrated by quantifying the extent of binding to parOP with competitor DNA relative to that seen in the absence of competitor (Fig. 5C, right panel). In contrast, the binding activities of both ParAWT and ParA351A to the nsDNA ligand parOpdel were equally sensitive to competition by excess nsDNA (Fig. 5, B and C, left panels). These data demonstrate that [ParA351A-ATP]* is less effectively competed away from parOP by nsDNA than ParAWT is competed by nsDNA. They support the idea that ParA351A behaves as a super-repressor because the pool of nonDNA bound [ParA-ATP]* in the cell is high enough to fully repress transcription from parOP without the need for ParB.

The ATP cycle of ParA explored by epistatis of mutations affecting repressor activity

We next examined the effect of the R351A mutation in vivo in relationship to other ParA mutations that are blocked in different steps by exploiting differences in their repressor activities in vivo. Our in vitro results place the defect caused by the R351A mutation after ATP binding and the ATP* conformational change, at the step of nsDNA binding, and before the formation of the NAC complex (Figs. 2 to 4). Two previously similar to previous experiments (27), we see that ParA351A was only modestly reduced for binding to parOP. The dissociation rate of ParA351A (3.3 × 10⁻² ± 7.7 × 10⁻⁵ s⁻¹) was also only modestly increased with respect to that of ParAWT (2.1 × 10⁻² ± 3.9 × 10⁻⁵ s⁻¹).

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ParA-DNA interactions during partition and expression

characterized mutations in the ATP-binding site are blocked at different steps, K122Q and K122R, and possess distinct repressor phenotypes in vivo (12, 21, 26). ParA\textsuperscript{K122Q} is a super-repressor because the mutation prevents the ParA-ATP to [ParA-ATP]* transition. ParA\textsuperscript{K122R} forms [ParA-ATP]* and assembles NAC complexes, but is blocked at NAC disassembly (26). It retains its basal and weak ATPase and repressor activities, which are insensitive to stimulation by ParB (26). Therefore our biochemical data placed the R351A defect between the steps blocked by K122Q and K122R, and we reasoned that double mutants would exhibit the phenotype of the earlier blocked step; that is, ParA\textsuperscript{K122Q,R351A} should behave as ParA\textsuperscript{K122Q}, and ParA\textsuperscript{K122R,R351A} should behave as ParA\textsuperscript{R351A}. We exploited the differences in repressor activity to test this prediction in vivo (Fig. 1). First, using the low-copy parOP-lacZ reporter system, parA\textsuperscript{R351A} was indeed epistatic to parA\textsuperscript{K122R}; that is, parA\textsuperscript{K122R,R351A} exhibited the super-repressor activity of parA\textsuperscript{R351A} rather than the weak repressor activity of parA\textsuperscript{K122R} (Fig. 1A). Second, we exploited the high-copy reporter system to discriminate between the super-repressor activities of parA\textsuperscript{K122Q} and parA\textsuperscript{R351A} (Fig. 1B). These two alleles have small but reproducible and diagnostic differences in their ability to repress in the presence and absence of ParB. Repression by parA\textsuperscript{R351A} is slightly stimulated by the presence of parB, but this stimulation, although small, is bypassed for the parA\textsuperscript{K122Q,R351A} double mutant (Fig. 1B). Therefore, parA\textsuperscript{K122Q} was epistatic to parA\textsuperscript{R351A}.

Discussion

In this work we have exploited the ParA\textsuperscript{R351A} protein to better understand the role of DNA binding in ParA actions during plasmid partition and gene expression. Our previous analyses showed that the conformational change that creates [ParA-ATP]* is necessary for nsDNA-binding activity and partition, and inhibitory to repressor activity. Here we separate the requirement for the conformational change from the nsDNA-binding activity, and examine how nsDNA binding affects ParA’s site-specific DNA-binding function.

ParA\textsuperscript{R351A} defines a novel class of ParA super-repressor driven by a shift in DNA binding preference

WT ParA is a weak repressor of par gene expression unless it is stimulated by the co-repressor ParB (2). The ParA super-repressor class of mutants created by mutations in its ATP-binding site (such as K122Q) bypassed this stimulation to create repressors that were fully “on” and were insensitive to further stimulation by ParB (21). Further analysis indicated that they were locked into the repressor form because they could not undergo the ParA-ATP to [ParA-ATP]* transition (12). The ParA\textsuperscript{R351A} protein presents a new ATP-binding site-independent modification to improve repression at parOP, specifically through an alteration in DNA binding preference. The R351A mutation alters the relative affinity between nsDNA and parOP without affecting the transition of ParA-ATP into the [ParA-ATP]* state (Figs. 4B and 5B). By impairing the [ParA-ATP]*-dependent binding to nsDNA, [ParA-ATP]* binding to parOP was enhanced. The simplest explanation is that reduced binding to nsDNA increases the free, unbound pool of [ParA-ATP]*, which favors ParA-parOP complex formation because [ParA-ATP]* is competent to bind to parOP. This is supported by the observation that the R351A mutation transformed para\textsuperscript{K122R} to a super-repressor in vivo (Fig. 1A). The effect of the R351A change is specific to the [ParA-ATP]* form, as the [ParA-ATP]-deficient para\textsuperscript{K122Q} allele is epistatic to para\textsuperscript{R351A} in the repression assay (Fig. 1B).

ParA binding to nsDNA is altered by the R351A mutation

The change in affinity and kinetics of ParA\textsuperscript{R351A} binding to nsDNA compared with that of ParA\textsuperscript{WT} supports the proposal that Arg\textsuperscript{151} is part of the nsDNA-binding site in ParA, consistent with the defects caused by the analogous mutations in F SopA (K341A) and B. subtilis Soj (R189E) (23, 30). The BLI results also point to a difference in the way that ParA\textsuperscript{WT} and ParA\textsuperscript{R351A} bind to nsDNA. Experiments done previously indicated that there was an apparent cooperativity of [ParA-ATP]* binding to DNA when measured by TIRF microscopy in vitro (12). Characterization of [ParA-ATP]* binding to nsDNA by BLI strongly supported the cooperativity, especially when contrasted against ParA\textsuperscript{R351A} (Fig. 4F). Most obvious at lower concentrations of ParA\textsuperscript{WT}, the binding of [ParA\textsuperscript{WT}-ATP]* to nsDNA appeared to be sigmoidal, with an acceleration in binding before slowing to saturation (Figs. 4B and Fig. S1A). This cooperativity in binding to nsDNA may reflect additional ParA-ParA interactions when the protein binds to nsDNA, suggesting that patches of ParA may stabilize binding on the bacterial nucleoid. In contrast, the association rate of [ParA\textsuperscript{R351A}-ATP]* on nsDNA appeared linear at low ParA concentrations and overall resembled a single-order exponential decay, suggesting a predominantly singular binding mode and a loss of cooperativity. The substantial increase in $k_{\text{off}}$ for [ParA\textsuperscript{R351A}-ATP]* on nsDNA is a simple explanation for the loss of apparent cooperativity. As dissociation events occur more frequently, cooperative [ParA-ATP]*-[ParA-ATP]* interactions on nsDNA are less likely to occur, reducing their ability to engage in or promote additional [ParA-ATP]* binding. Although it is formally possible that the R351A mutation affects cooperativity directly by altering ParA dimer-dimer interactions, we think that this explanation is unlikely given the properties of mutations in the analogous C-terminal regions of other ParAs (23, 30, 39).

Cooperativity of ParA-DNA association is proposed to be important for ParA function in recent models of plasmids F and TP228 (16, 17). The idea is that concentrated patches or a meshwork of ParA serves as a sink of ParA to transiently anchor ParB-parS plasmid complexes. For example, when examined in vivo, F plasmid SopA localization is enriched at so-called high-density chromosomal regions, and plasmids are proposed to move from one high-density chromosomal region to another during partition (16).

Kinetics of nsDNA binding is fundamental to partition activity

ParA binding nonspecifically to the bacterial nucleoid is a key property of the Brownian ratchet mechanism of partition. In response to ParB-parS-plasmid complexes, ParA forms
ParA readily exchanges ADP (ParB/operative binding of [ParAR351A-ATP]* removes the preferential binding to [ParAR351A-ATP]*-nucleoid regions, also reducing the [ParAR351A-ATP]* conformation to [ParA-ATP]*, which in turn promotes binding to nsDNA. The latter step represents binding to the bacterial nucleoid. Repression at parOP depends on the contributions of ParA-ADP, ParA-ATP, and [ParA-ATP]* in the cell. Although [ParA-ATP]* is likely the predominant form inside cells, its nsDNA-binding activity predominates so that little is available to bind parOP. The role of ParB in this process is to help drive redistribution of ParA via ATP hydrolysis or direct effects on ParA conformation. The R351A change, however, reduces nsDNA binding so that [ParAR351A-ATP]* is able to effectively act as a repressor at parOP without the stimulatory effect of ParB. 8. model for [ParA-ATP]* cycling on the nucleoid and the consequence of the R351A mutation. As ParB-plasmid complexes promote ParA hydrolysis and dissociation from the nucleoid (upper panel), ParA readily exchanges ADP (blue) for ATP (red) and undergoes a slow transition to [ParA-ATP]* (purple). [ParA-ATP]* has preferential binding for [ParA-ATP]*-nucleoid regions (cooperativity). Binding of [ParA-ATP]* on the nucleoid is stable, and dissociation events are infrequent. These binding properties keep [ParA-ATP]* concentration ahead of the migrating ParB-plasmid complex, and low in its wake after stimulated hydrolysis, establishing a strong migratory gradient. The R351A mutation disrupts the cycle by altering ParA-nucleoid equilibria (lower panel). The reduced affinity of [ParAR351A-ATP]* for the nucleoid redistributes much of the bound [ParAR351A-ATP]* to an unbound state. This increases the replenishment of ParA onto the nucleoid after it has been ejected by ParB. The damaged cooperative binding of [ParAR351A-ATP]* removes the preferential binding to [ParAR351A-ATP]*-nucleoid regions, also reducing the [ParAR351A-ATP]* concentration ahead of the ParB-plasmid, and compromising ParB-plasmid migration. Weights of the arrows in both panels indicate relative rates of occurrence of the transitions.

Figure 6. ParAR351A redistributes the [ParA-ATP]*-nsDNA balance to alter repression and partition. A, the ATP cycle of ParA. ParA binds ATP, which promotes the conformational change to [ParA-ATP]*, which in turn promotes binding to nsDNA. The latter step represents binding to the bacterial nucleoid. ParB-parS plasmid DNA associates with ParA and the nucleoid, after which ParB promotes ParA dissociation from nsDNA, shown here as release of ParA-ADP. ParB-parS reassociate with adjacent ParA bound to the nucleoid, and the cycling of this activity represents movement of the plasmid over the ParA-bound nucleoid. Repression at parOP depends on the contributions of ParA-ADP, ParA-ATP, and [ParA-ATP]* in the cell. Although [ParA-ATP]* is likely the predominant form inside cells, its nsDNA-binding activity predominates so that little is available to bind parOP. The role of ParB in this process is to help drive redistribution of ParA via ATP hydrolysis or direct effects on ParA conformation. The R351A change, however, reduces nsDNA binding so that [ParAR351A-ATP]* is able to effectively act as a repressor at parOP without the stimulatory effect of ParB. B, model for [ParA-ATP]* cycling on the nucleoid and the consequence of the R351A mutation. As ParB-plasmid complexes promote ParA hydrolysis and dissociation from the nucleoid (upper panel), ParA readily exchanges ADP (blue) for ATP (red) and undergoes a slow transition to [ParA-ATP]* (purple). [ParA-ATP]* has preferential binding for [ParA-ATP]*-nucleoid regions (cooperativity). Binding of [ParA-ATP]* on the nucleoid is stable, and dissociation events are infrequent. These binding properties keep [ParA-ATP]* concentration ahead of the migrating ParB-plasmid complex, and low in its wake after stimulated hydrolysis, establishing a strong migratory gradient. The R351A mutation disrupts the cycle by altering ParA-nucleoid equilibria (lower panel). The reduced affinity of [ParAR351A-ATP]* for the nucleoid redistributes much of the bound [ParAR351A-ATP]* to an unbound state. This increases the replenishment of ParA onto the nucleoid after it has been ejected by ParB. The damaged cooperative binding of [ParAR351A-ATP]* removes the preferential binding to [ParAR351A-ATP]*-nucleoid regions, also reducing the [ParAR351A-ATP]* concentration ahead of the ParB-plasmid, and compromising ParB-plasmid migration. Weights of the arrows in both panels indicate relative rates of occurrence of the transitions.

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Dynamic gradients on and in the nucleoid, which move with the associated plasmids. The gradients form because ParB stimulates low ahead of the migrating ParB-plasmid complex, and low in its wake after stimulated hydrolysis, establishing a strong migratory gradient. The R351A mutation disrupts the cycle by altering ParA-nucleoid equilibria (lower panel). The reduced affinity of [ParAR351A-ATP]* for the nucleoid redistributes much of the bound [ParAR351A-ATP]* to an unbound state. This increases the replenishment of ParA onto the nucleoid after it has been ejected by ParB. The damaged cooperative binding of [ParAR351A-ATP]* removes the preferential binding to [ParAR351A-ATP]*-nucleoid regions, also reducing the [ParAR351A-ATP]* concentration ahead of the ParB-plasmid, and compromising ParB-plasmid migration. Weights of the arrows in both panels indicate relative rates of occurrence of the transitions.

ParA-R351A allowed us to specifically scrutinize the role of DNA binding following the [ParA-ATP]* transition. Our results here show that the R351A mutation does not alter the interactions of ParA with ATP or its ability to undergo the conformation change to [ParA-ATP]* (Fig. 2), and impairs the next step, binding to nsDNA (Fig. 6A). We conclude that this defect is responsible for the defect in NAC assembly (Fig. 3); that is, the nsDNA-binding activity of ParA is necessary to form these complexes in vitro. NAC complex assembly by ParAR351A can be partially restored by increasing the concentrations of ParA and/or ParB (Fig. 3), suggesting that it is also limited by the availability of ParA molecules able to interact directly with ParB.

Altering the kinetics, affinity, and apparent cooperativity of the nsDNA-binding activity of ParA would alter the density and dynamics of ParA gradients that form on the nucleoid. First, a lower affinity and cooperativity of ParA would provide fewer localized ParA molecules on the nucleoid available to interact with ParB molecules bound to parS, and could weaken the plasmid association with ParA on the nucleoid. Second, a high off-rate of ParA binding kinetics would change the
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dynamics of the ParA gradients, which rely on the slow refilling of areas of the nucleoid that are depleted of ParA after ejection by ParB. The slow refilling depends on the slow ParA-ATP to [ParA-ATP]* transition as well as slow off rates from DNA that limit diffusion along the DNA surface (12, 13). ParA<sub>R351A</sub>, whereas unaltered for the [ParA-ATP]* transition, exhibited a substantially faster dissociation rate from DNA, which should then substantially increase the refilling rate because nucleoid-bound [ParA<sub>R351A</sub>-ATP]* dissociates and rebinds more frequently to quickly replenish any localized depletion (Fig. 6B).

Our experimental results and conclusions are supported by mathematical models of this mechanism, which illustrate the importance of nsDNA binding and its parameters on the mechanism. Hu et al. (40, 41) modeled conditions that drive ParB-plasmid migration over a “carpet” of ParA molecules in the context of defined variables: ParB complex size and density, ParA density, association and dissociation rates of ParA-ParB bonds, and the length and force of ParA-ParB bonds. The model demonstrated the importance of replenishment of [ParA-ATP]* on the carpet. As [ParA-ATP]* replenishment rates increase because there is more available free [ParA-ATP]* as with ParA<sub>R351A</sub>, the effective local gradient of [ParA-ATP]* would be reduced (Fig. 6B).

P1 ParA acts both as the driver for plasmid partition and the repressor of the par genes, mediated by nonspecific and site-specific DNA-binding activities, respectively. ParA interactions with ATP have been finely tuned to promote and regulate both activities. The current results with ParA<sub>R351A</sub> illustrate the importance of the balance of DNA-binding activities in both roles. ParB also plays important roles in mediating this balance by its activity as a corepressor. Although P1 partition does not require the repressor activity of ParA (20), and the repressor activity does not require partition (2, 21), these two roles are not independent and occur concurrently inside the cell. The presence of parR, either in cis or in trans to parOP, increases repression by ParA in vivo (42), consistent with a role for ParB in converting the nsDNA-binding form of ParA to its site-specific DNA-binding form when ParB stimulates ParA release from the nucleoid during partition (Fig. 6).

Recent evidence has added a new nucleotide cofactor to partition with the demonstration that ParB binds and hydrolyses CTP (43–45). Furthermore, the evidence indicates that CTP, which binds to the N-terminal domain of ParB, allows ParB to clamp onto DNA when it loads at parS, and facilitates loading multiple ParB molecules. Large ParB-parS partition complexes are critical to partition models (41), because they provide a high local concentration of ParB molecules to interact with the density of ParA molecules on the bacterial nucleoid. In this model, CTP facilitates ParA-ParB interactions by promoting a high local concentration of ParB at parS. Similarly, ATP could facilitate ParA-ParB interactions by promoting cooperative ParA-DNA clusters on the nucleoid, a step that is damaged by the R351A substitution in ParA. That CTP is exploited for ParB-DNA interactions and ATP for ParA-DNA interactions is an intriguing division of labor, and dissection of the complicated interplay among protein, nucleotide and DNA molecules will be essential to fully understand the biological patterning promoted by ParA and ParB proteins.

Experimental procedures

Bacterial strains, plasmids, and growth media

E. coli DH5 (recA1) was used for plasmid maintenance, cloning, and plasmid stability assays. E. coli DH5<sub>Δlac</sub> was used as a lacZ reporter assay. Plasmids used in this study are described in Table 1. Bacteria were grown in Luria-Bertani (LB) media. Antibiotics utilized were ampicillin (Ap; 100 μg/ml) and/or chloramphenicol (Cm; 25 μg/ml).

Reaction buffers

Buffer B was 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 20% (v/v) glycerol. Buffer BI was 1× PBS with 10 μg/ml of bovine serum albumen and 12.5 parts per million Tween-20. When nucleotide is present, Buffer BI is also supplemented with 5 mM MgCl<sub>2</sub> and 1 mM ADP or ATP.

Plasmid stability assays

The miniP1 plasmid pBEF246 (par<sup>S</sup>+, Cm<sup>R</sup>) is unstable because it lacks both parA and parB (33). WT or mutant parA and parB were provided in trans on compatible pBR322 derivatives (Ap<sup>R</sup>, see Table 1). Fresh colonies of E. coli DH5 cells containing miniP1 pBEF246 and pEF5 (parA<sup>+</sup> parB<sup>+</sup>), pBEF346 (parA<sub>R351A</sub> parB<sup>+</sup>), or the vector pBR322, were inoculated into LB medium with Ap, grown overnight at 37 °C (~15–18 generations), and then diluted and plated onto LB agar plates with Ap. Resultant colonies were transferred with toothpicks to LB agar plates containing Ap or Cm to determine plasmid retention. A minimum of 200 colonies were tested for each strain, and each assay was repeated at least three times. Plasmid retention was calculated as the percent of Ap<sup>R</sup> Cm<sup>R</sup> colonies in the Ap<sup>R</sup> population.

ParA repressor assays

Liquid β-gal assays were carried out following the protocol by Zhang and Bremer (46), using both low- and high-copy reporter systems that have been previously described (21). Briefly, the low-copy reporter system contains parOP-lacZ on a λ phage integrated into the E. coli chromosome, and the high-copy reporter contains parOP-lacZ cloned into the vector pST52 (pBEF239, Table 1), with or without parB were provided in trans under the control of a modified β-lactamase promoter in the vector pBR322 (pEF8 and derivatives, Table 1). Cells were grown in LB medium with Ap at 37 °C until A<sub>600</sub>approximate 0.3, and 20 μl of culture was fixed with permeabilization solution for use in the assay. Each assay was repeated with three technical replicates for each of three biological replicates.

Proteins and ATPase assays

P1 ParA (WT and ParA<sub>R351A</sub> alleles) and ParB were purified as described previously (22, 26). ATPase activity was measured in 10 mM Tris HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.5 mM [γ-<sup>32</sup>P]ATP, 100 μg/ml of BSA, and 100 μg/ml of sonicated salmon sperm DNA (s<sup>3</sup>-DNA). Samples were incubated for 90 min at 30 °C and analyzed by TLC as described previously (21, 26).
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Fluorescence assays

Tryptophan fluorescence assays were carried out similarly to those done previously (12). Measurements were carried out in triplicate at an excitation wavelength of 297 ± 1.25 nm, and emission wavelength was monitored at 340 ± 1.25 nm in a T-format Photon Technology International spectrofluorimeter at room temperature (22 °C). Reactions were initiated by mixing ParA and s3-DNA in Buffer B, and baseline fluorescence was measured for 60 s. The sample was removed from the spectrofluorimeter, ATP was added, and the sample rapidly mixed (in ~5 s) and returned to the spectrofluorimeter. Fluorescence was followed for an additional 300 s. The fluorescence signal was normalized to that before the addition of ATP, correcting for dilution. Final concentrations of each reaction were 2 μM ParA, 100 μg/ml of s3-DNA, and 1 mM ATP.

DNA-binding assays of ParA to Alexa514-labeled DNA were performed as described previously (12) and as described above for tryptophan fluorescence assays. Measurements were carried out in triplicate at an excitation wavelength of 513 ± 1.25 nm, and emission wavelength was monitored at 540 ± 1.25 nm. Final concentrations of each reaction mixture were 2 μg/ml of Alexa 514-labeled DNA, 1 mM ATP, and ParA as indicated in each figure.

Light-scattering assays

Light-scattering assays were carried out as described previously (25). Briefly, assays were carried out in quadruplicate on a T-format Photon Technology International fluorometer at 25 °C with excitation and emission monochromators set to 467 ± 1.25 nm. Samples containing ParA, ParB, 50 μg/ml of s3-DNA, and 0.1 mg/ml of α-casein were manually mixed in Buffer B, and scattered light was measured for 30 s. ATP was prepared separately in Buffer B with 0.1 mg/ml of α-casein, rapidly mixed (~5 s) with the ParA/ParB/s3-DNA solution, and scattered light was immediately measured for 300 s.

Biolayer interferometry assays

Biotinylated DNA substrates for BLI were prepared by PCR with one biotinylated primer and one nonbiotinylated primer: NSB is a 348-bp PCR product internal to the coding sequence of parB, amplified from pEF8 with primers 5′-GTCTGCTGGTGTAGCAGATG and 5′-[Biotin]′-CTTTATCCGTGAGTAGTGGAC; parOpB is a 348-bp PCR product containing parOp, amplified from pBEF101 with primers 5′-CATCGT-GAAGCTTTGGCTGC and 5′-[Biotin]′-AACCTCGTGGCAGTGAGTCTC; parOpDelB is a 353-bp PCR product from pBEF109, which was derived from pBEF101 by deleting most of the parOp sequence including residues shown previously to be necessary for ParA repressor function (3) (Table 1). It was amplified with primers 5′-GTTTAATTTGCTCATGACGG and [Biotin]′-AAC-TCGTCCGCGTGCTGC; PCR products were separated by agarose gel electrophoresis followed by purification with the Qiagen QIAquick Gel Extraction Kit, and quantified by A260.

FortéBio Dip and Read™ streptavidin biosensors were prepared by preincubation in 1× PBS, 5 mM EDTA for ~30 min, followed by incubation with 15 nM purified PCR product in 1× PBS, 5 mM EDTA for 500 s, and washed free with 1× PBS, 5 mM EDTA for at least 300 s (or until baseline was reached). At least one DNA-free streptavidin control biosensor was prepared for every three loaded biosensors used in analysis. Prepared biosensors were equilibrated in Buffer BI with MgCl2 and nucleotide to establish a baseline for 60 s, then moved to wells containing protein dilutions in Buffer BI with MgCl2 and nucleotide to assess ParA binding, followed by dissociation of ParA in Buffer BI with MgCl2 and nucleotide (all ParA concentrations and association/dissociation times indicated in figures). The presence of competing nsDNA (s3-DNA) in either association or dissociation steps is indicated in the respective figures. Binding responses were normalized against substrate-free control biosensor(s), and a baseline response of zero was established by the end point response of the baseline wash (average of last 5 s), with the Octet System Data Analysis Software version 7.1. Calculation of koff values was performed with a nonlinear one-phase decay fit in GraphPad Prism version 8.01.

Data availability

All data are contained within the article.

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Abbreviations—The abbreviations used are: nsDNA, nonspecific DNA; BLI, biolayer interferometry; NAC, nucleoid adaptor complex; TIRF, total internal reflection fluorescence; wHTH, winged helix-turn-helix; Ap, ampicillin; Cm, chloramphenicol; s3-DNA, sonicated salmon sperm DNA.

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