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Competing ParA Structures Space Bacterial Plasmids Equally over the Nucleoid

Robert Ietswaart1*, Florian Szardenings2*, Kenn Gerdes2,3, Martin Howard1*

1 Computational and Systems Biology, John Innes Centre, Norwich, United Kingdom, 2 Centre for Bacterial Cell Biology, Newcastle University, Newcastle upon Tyne, United Kingdom, 3 Department of Biology, University of Copenhagen, Copenhagen, Denmark

Abstract

Low copy number plasmids in bacteria require segregation for stable inheritance through cell division. This is often achieved by a parABC locus, comprising an ATPase ParA, DNA-binding protein ParB and a parC region, encoding ParB-binding sites. These minimal components space plasmids equally over the nucleoid, yet the underlying mechanism is not understood. Here we investigate a model where ParA-ATP can dynamically associate to the nucleoid and is hydrolyzed by plasmid-associated ParB, thereby creating nucleoid-bound, self-organizing ParA concentration gradients. We show mathematically that differences between competing ParA concentrations on either side of a plasmid can specify regular plasmid positioning. Such positioning can be achieved regardless of the exact mechanism of plasmid movement, including plasmid diffusion with ParA-mediated immobilization or directed plasmid motion induced by ParB-parC-stimulated ParA structure disassembly. However, we find experimentally that parABC from Escherichia coli plasmid pB171 increases plasmid mobility, inconsistent with diffusion/immobilization. Instead our observations favor directed plasmid motion. Our model predicts less oscillatory ParA dynamics than previously believed, a prediction we verify experimentally. We also show that ParA localization and plasmid positioning depend on the underlying nucleoid morphology, indicating that the chromosomal architecture constrains ParA structure formation. Our directed motion model unifies previously contradictory models for plasmid segregation and provides a robust mechanistic basis for self-organized plasmid spacing that may be widely applicable.

Introduction

parABC loci generate equally spaced positioning of many bacterial low copy number plasmids, thereby ensuring stable plasmid inheritance [1]. However, the underlying mechanism of action is not satisfactorily understood. In contrast, plasmid segregation mediated by actin homolog ParM is increasingly well explained and involves filaments that push plasmids apart in a mitotic-like process [2]. Understanding of the parABC mechanism is important, as it belongs to the most common class of DNA segregation systems in prokaryotes, used by chromosomes and antibiotic-resistance-carrying plasmids [1,3–5]. Moreover, it is used in other conceptually similar processes, such as chemotactic cluster positioning and partitioning of carbon-fixing carboxysomes [6,7].

The parABC locus present in Escherichia coli plasmids such as pB171 and P1 encodes two proteins: ParA, a P-loop ATPase that binds DNA non-specifically in its dimeric ATP-bound form (ParA-ATP for short) [8,9], and the DNA-binding protein ParB that binds site-specifically to the parC region [10,11]. Fluorescence microscopy has provided evidence for ParA movement over the nucleoid with spatiotemporal oscillations in helix-like structures [12–14]. ParB and parC are required for these dynamics [12], with ParB promoting the conversion of ParA-ATP to dimeric ParA-ADP (ParA-ADP for short), causing ParA to unbind from the nucleoid [8,9]. The time period required for nucleoid-disassociated ParA to regain the ability to bind the nucleoid is sufficiently long in vitro to ensure that the relative locations of ParA-ADP unbinding and later ParA-ATP rebinding would be uncorrelated due to cytoplasmic ParA diffusion [8]. However, once nucleoid-bound, whether Par-A-ATP then polymerizes to form long filaments in vivo is currently controversial. Furthermore, the means by which plasmids move under the influence of ParA, and whether ParA polymerization is important for this movement, are also unclear. Nevertheless, the outcome of these ParA dynamics in E. coli is equally spaced positioning of plasmid foci over the nucleoid [9,13–15]. This state is achieved regardless of the plasmid focus number n_f or cell length, with plasmid foci repositioned in the wake of retracting ParA structures [9].

Several mechanisms have been proposed to explain ParA-mediated plasmid movement. One hypothesis proposes that ParA-ATP polymerizes on the nucleoid to form long filaments and that...
plasmid translocation is achieved by ParB-stimulated retraction of the polymers, generating effective plasmid-pulling [3,9]. Other proposals are based on ParA-ATP forming a gradient-like distribution on the nucleoid, without a necessity for polymerization [8,16–21]. It is currently unclear whether any of these mechanisms can explain equal plasmid spacing given the known physiological and biochemical constraints. Here, we therefore investigate which aspects of the polymer and gradient mechanisms are required and sufficient to explain the observed plasmid translocation and equal spacing over the nucleoid.

We begin by showing mathematically that competition between dynamic ParA concentrations on either side of a plasmid can lead to equal plasmid spacing. This mechanism relies on an ability of a plasmid to move towards higher ParA concentrations, but the exact means of such movement is not important. We then investigate theoretically specific means of plasmid movement and examine whether predictions from such models are borne out experimentally. We define a computational diffusion/immobilization model where nucleoid-bound ParA-ATP can anchor diffusing plasmids. We show that diffusion/immobilization can in principle space mobile plasmids equally over the nucleoid. However, experiments measuring increased plasmid mobility in the presence of the pB171 parABC locus (par2), lead us to disfavor this model. Instead we favour a directed motion mechanism in which ParA structure formation provides directionality to plasmid motion thereby speeding up plasmid movement. The directed motion model produces robust equal plasmid spacing with, on average, relatively symmetric ParA distributions, a prediction we verify experimentally. Furthermore, we show experimentally that ParA organization is dependent on the underlying nucleoid structure, with nucleoid disruption resulting in perturbed plasmid positioning. Our combination of modeling and experiments has for the first time uncovered a robust mechanism for plasmid spacing that unifies previous proposals.

Results
ParB-GFP foci are spaced equally over the nucleoid
To study par2-mediated plasmid segregation, we investigated ParB-GFP localization, expressed from a par2-carrying mini-R1 test plasmid. The par2 locus containing the parB::sfGFP fusion is fully functional as judged by loss-frequency assays (S1A Fig.). As previously described, usage of ParA-GFP and the tetO-TetR-mCherry labeling system also does not affect plasmid stability, indicating full functionality [9,12]. ParB-GFP forms foci that are regularly positioned along the long cell axis in vivo (Fig.1A), consistent with ParB-binding to plasmid-encoded parC regions [10,11]. Since plasmid dynamics occur primarily over the nucleoid, we reasoned that plasmid positioning with respect to the nucleoid rather than cell length is most informative. Therefore we measured ParB-GFP foci localization, together with Hoechst (DNA) stain to determine the nucleoid boundaries. As expected ParB-GFP foci colocalized exclusively with the Hoechst stain, and were equally spaced over the nucleoid (Fig. 1B,C,D for np = 1,2, S2A,B Fig. for np = 3,4).

Mathematical analysis shows that dynamic, competitive ParA concentrations can generate equal plasmid spacing
Several studies have proposed that plasmid positioning is controlled by a concentration gradient of ParA over the nucleoid [8,16–20]. Intuitively in this mechanism, ParB bound to plasmid parC (ParB-parC complex) interacts with nucleoid associated ParA-ATP, which effectively anchors the plasmid to the nucleoid. At the same time, the ParB-parC complex stimulates ParA-ATP hydrolysis causing a local ParA-ATP depletion. These processes could then generate a ParA-ATP gradient which a plasmid is able to follow. Reorganization of ParA gradients under the influence of multiple ParB-parC complexes might then lead to equal plasmid spacing. To rigorously understand if, and with what requirements, equal spacing can be achieved we develop here a minimal mathematical model based on the above principles.

We model the nucleoid as a 1d system of length L (along the long axis of the cell) on which ParA-ATP and plasmids can interact. Let A(x,t) denote the nucleoid-associated ParA-ATP concentration at position x relative to one nucleoid edge at time t. Let x1(t),..xnp(t) be the positions of the np plasmids. ParA can bind to the nucleoid with flux J. Once bound, ParA-ATP can diffuse along the nucleoid with diffusion constant D. For simplicity, we first assume that the ParA-ATP concentration at each plasmid is zero due to a high ParA-ATP hydrolysis rate. Later on we will relax this assumption. This system can be described by the deterministic reaction-diffusion equations:

\[
\frac{\partial A(x,t)}{\partial t} = D \frac{\partial^2 A(x,t)}{\partial x^2} + J
\]

Boundary Conditions : \[ A(x; t) = 0 \text{ for } 1 \leq i \leq np \]

\[
\frac{\partial A(x,t)}{\partial x} \bigg|_{x=0} = 0 = \frac{\partial A(x,t)}{\partial x} \bigg|_{x=L} \text{ for all } t.
\]

We now use separation of time scales to obtain the steady-state solution for A(x); we assume that plasmid motion is much slower than the time for individual ParA-ATP molecules to diffuse over the nucleoid and generate a concentration profile. In this way, the plasmid positions x1,...xp are effectively time-independent and a priori unknown. The equation for A(x) then simplifies to:

\[
\frac{d^2 A(x)}{dx^2} = -J L D
\]

Boundary Conditions : \[ A(x; t) = 0 \text{ for } 1 \leq i \leq np \]

\[
\frac{d A(x)}{dx} \bigg|_{x=0} = 0 = \frac{d A(x)}{dx} \bigg|_{x=L}.
\]

This equation can be solved by integrating twice using the boundary conditions. The solution is given by:

Author Summary
How DNA is stably inherited through cell division is a fundamental question in cell biology. The most common system that mediates plasmid DNA inheritance in bacteria is through a parABC locus, encoding proteins ParA and ParB, and DNA sequence parC. These components can position plasmids at equally spaced positions throughout a cell to ensure plasmids are present in both daughter cells when the cell divides into two. Here we study the mechanism by which ParA structures achieve this precise positioning. We show that ParA can direct relatively immobile plasmids over the bacterial chromosome using self-organizing, competitive ParA structures, whose disassembly is induced by plasmid parC-bound ParB. More generally these findings will help us to understand transport and regular positioning of intracellular cargo.
Next we use these equations to compute the diffusive fluxes of ParA-ATP, $j_i = |D \frac{dA}{dx}|$ at a plasmid location $x_i$, where the + and - superscripts below refer to the flux from the right (+) and left (-) respectively. We find:

$$j_i^+ = j_{i+1}^+ = \frac{J (x_{i+1} - x_i)}{2L},$$

$$j_i^- = j_{i+1}^- = \frac{J (x_{i+1} + x_i)}{2L},$$

$$j_{np}^- = \frac{J (L - x_{np})}{L}.$$

Clearly, a symmetric ParA concentration profile, where fluxes from either side balance, is only possible for $x_i = L - x_{np} = \frac{1}{2} (x_{i+1} - x_i)$. The plasmids are then equally distributed with $x_i = \frac{L}{2n_p} + \frac{L}{n_p} (j - 1)$. We note that the predicted inter-plasmid spacing $L/n_p$ arising from this analysis is consistent with our experimental findings (Fig. 1D, S2B).

Importantly, the above analysis provides insight into the equal spacing mechanism. The key is that the above fluxes depend on the distances either between the plasmid and nucleoid end, or between neighboring plasmids. This feature is a consequence of ParA binding to the nucleoid anywhere, but with ParA release only occurring at a plasmid. In order for these on and off fluxes to balance at steady-state, the off-flux at a plasmid must scale with the inter-plasmid or plasmid-nucleoid-end distance. In this way, non-local information about lengths is converted into local spacing information encoded in the slope of ParA-ATP concentration. For non-equal plasmid spacing, the competing ParA concentrations on either side of a plasmid will be unequal, with one gradient steeper than the other. The steeper gradient corresponds to the side with...
the greater available space for ParA binding. If a plasmid can preferentially move (on the appropriate slow time scale) towards the side with the locally steepest ParA-ATP concentration, the plasmids are then progressively restored towards equal spacing. As this process occurs, the ParA-ATP concentrations will dynamically reorganize such that a symmetric configuration around a plasmid is reached only when the plasmids are equally spaced. In this state, where the competing ParA-ATP concentrations are symmetric, plasmid movement would no longer have a directional preference and would thus remain, on average, stationary.

So far, we have assumed that the ParA-ATP concentration vanishes at a plasmid, corresponding to very fast ParA-ATP hydrolysis. However, our results also hold true when we only assume that this hydrolysis occurs with a finite rate $k_B$, leading to a non-zero concentration of ParA-ATP at a plasmid. This ParA-ATP can then anchor a plasmid to the nucleoid before being hydrolysed. This more general and realistic case is presented in the S1 Text, but our overall conclusions reached above remain unchanged.

From the above analysis, we see that the following conditions are required for equal plasmid spacing: (1) movement of a plasmid towards higher ParA-ATP concentrations. (2) diffusion of (at least a fraction of) ParA-ATP over the nucleoid to ensure formation of competitive concentration gradients. Single molecule tracking experiments in vitro support this assumption [17,18]. (3) ParA-ATP hydrolysis must occur (predominantly) by plasmid-associated ParB-parC complexes, again to ensure gradient formation. (4) ParA-ATP must adopt a 1d-like configuration, as previously claimed [9,13,14]. If ParA were not organized in this fashion, it would be possible for ParA to diffuse around the sides of a plasmid without encountering the hydrolyzing effect of the ParB-parC complex. This would equalize the ParA concentrations on both sides even in the case of asymmetrically placed plasmids, leading to failure of the equal spacing mechanism. This assumption is in line with our subsequent experiments (see below). Due to this proposed 1d-like nature, we will from now on refer to the ParA distributions away from a plasmid as ParA structures. (5) There must be a separation of time scales between plasmid movement and ParA concentration reorganization, as discussed above.

Importantly, this overall mechanism is not reliant on a specific type of plasmid translocation. Any process that would allow a plasmid to move into regions of higher ParA concentration will suffice. In the following sections we therefore analyze different means of plasmid movement and compare them with our experimental data to determine which is used in our par2 segregation system.

**Diffusion/immobilization model could space highly mobile plasmids equally over the nucleoid**

In the previous section the mechanistic details of plasmid movement towards a higher ParA concentration were not specified. We now examine a specific implementation involving a diffusion-immobilization mechanism. Using a minimal modeling approach, we assume that nucleoid-associated ParA-ATP can immobilize freely diffusing plasmids through its interaction with the ParB-parC complex and that ParA-ATP does not polymerize (Fig. 2A). Since the plasmid will tend to become immobilized in regions of higher ParA-ATP concentration, this process allows for effective plasmid translocation up a ParA-ATP concentration gradient. We also incorporate ParB-parC-stimulated ParA-ATP hydrolysis at a plasmid, in accordance with prior experimental data. To further investigate this mechanism, given the known physiological and biochemical constraints, we developed stochastic simulations using a Gillespie algorithm [22]. Here we use standard diffusion for the plasmid movement; below we discuss the potential impact of subdiffusive motion.

In our simulation, a one dimensional lattice with sites of size dx = 5 nm represents the nucleoid. ParA-ATP and plasmids can diffuse on the lattice with diffusion coefficient $D_A$ and $D_p$ respectively. Up to 35 ParA-ATP can bind to a plasmid at the same site with reaction parameter $k_{BA}$ reflecting the binding interaction of ParA-ATP and the ParB-parC complex [11]. More than one ParA-ATP bound to a plasmid reduces the plasmid diffusion constant to zero. Plasmid-bound ParA-ATP can be hydrolysed with reaction parameter $k_h$. Whenever a ParA-ATP hydrolysis event occurs, ParA unbinds from the nucleoid and becomes a cytoplasmic ParA-ADP. ParA-ADP can then be converted into a cytoplasmic ParA-ATP that is competent in DNA binding (cytoplasmic ParA-ATP for short) with a slow reaction parameter $k_{o}$ [8]. Cytoplasmic ParA-ATP can then bind anywhere along the nucleoid with parameter $k_{on}$ (see Materials and Methods and Tables 1,2 for details).

Prior work has demonstrated plasmid displacement along the long cell axis of up to 3–4 μm within 10 min [9,15]. With a diffusion/immobilization mechanism all plasmid movement in between immobilization events is generated by (unbiased) free diffusion, for which we have (in 1d) a mean square displacement (MSD) of $\langle r^2(t) \rangle = 2D_{pt} t$. By inserting the above length and time scales into this equation, we conclude that a plasmid diffusivity of at least $D_p \sim 10^{-2} \mu m^2 s^{-1}$ would be required to generate sufficiently rapid diffusive movement in accordance with previous experiments. We therefore chose $D_p = 10^{-1} \mu m^2 s^{-1}$. In order to physically justify that ParA can immobilise the plasmids, we chose the nucleoid bound ParA-ATP diffusivity to be lower than $D_p$, with $D_A = 10^{-2} \mu m^2 s^{-1}$ (Table 2). We experimentally constrained the overall copy number of ParA for pB171 par2 by semi-quantitative Western blots, which revealed that there were approximately $8 \times 10^3$ ParA monomers per cell (S1B Fig.). This diffusion/immobilization model could produce equal plasmid spacing on simulated growing nucleoids with varying numbers of plasmids (Fig. 2B,C, S3A). This result demonstrates that using a sufficiently high (low) plasmid (ParA) diffusivity, respectively, the equal plasmid spacing seen in our experiments (Fig. 1B,C,D, S2A,B Fig.) and previously [9], could in principle be achieved using a diffusion/immobilization mechanism.

**Free plasmid mobility is too low for a diffusion/immobilization mechanism**

To test whether the requirement of a relatively high free plasmid mobility is met in vivo, we compared the movement of test-plasmids with and without par2. We analyzed trajectories of labeled plasmid foci using the tetO-TetR-mCherry labeling system, measuring the positions over time (Fig. 3A) and MSDs for each time lag t. Plasmid motion will be biased by a functional par2+ partitioning system, in contrast to the random motion of par+. Nevertheless comparing MSDs can still be informative in comparing relative overall mobilities. On time scales up to a minute we found that the par2+ MSD is higher than in par+ (Fig. 3B), showing that, on average, par2+ plasmids are more mobile than their par+ counterparts. Note that the number of data points for the short time lags far exceeds the number of trajectories ($n_{par+} = 747$, $n_{par2+} = 763$), since every trajectory contains multiple short time lags. Consequently our estimates for the mean are relatively precise for short time lags. It is true that the error on the mean does not reflect inaccuracy due to experimental limitations in determining the actual plasmid position, for instance due to a finite pixel size. However, that error is the same for both par2+ and par+. Moreover, since the error is also time lag independent, it...
is taken into account in our fitting procedure as a time lag independent term (for more details see below and Materials and Methods). Overall, these results are hard to reconcile with a diffusion/immobilization mechanism where the par2 system can only immobilize plasmids, and thus lower their MSD. These MSD values could in principle be limited due to cellular confinement. However, we found that MSD saturation only starts to occur at much larger length scales at times of up to 10 min (Fig. 3C). In the

Table 1. Reactions and propensities used in the diffusion/immobilization model.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Propensities p_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_i → A_{i+1}, i = 0...L-2</td>
<td>( \frac{D_A}{dx^2} A[i] )</td>
</tr>
<tr>
<td>A_{i+1} → A_i, i = 0...L-2</td>
<td>( \frac{D_A}{dx^2} A[i+1] )</td>
</tr>
<tr>
<td>( P_{j,i} \rightarrow P_{j+1,i}, i = 0...L-2, j = 0...35 )</td>
<td>( \frac{D_p}{dx} P[j][i], \text{ if } j = 0 ) ( \frac{D_p}{dx} P[j][i], \text{ if } j = 1 ) ( 0, \text{ if } j &gt; 1 )</td>
</tr>
<tr>
<td>( P_{j,i} \rightarrow P_{j+1}, i = 0...L-2, j = 0...35 )</td>
<td>( \frac{D_p}{dx} P[j][i], \text{ if } j = 0 ) ( \frac{D_A}{dx} P[j][i], \text{ if } j = 1 ) ( 0, \text{ if } j &gt; 1 )</td>
</tr>
<tr>
<td>A_i + A_{i+1} \rightarrow A_{i+2}, i = 0...L-1, j = 0...34</td>
<td>k_{AB} P[j][i], A[i]</td>
</tr>
<tr>
<td>A_{i+1} \rightarrow A_i + A_{i+1}, i = 0...L-1, j = 0...34</td>
<td>k_B P[j+1][i]</td>
</tr>
<tr>
<td>A_{ADD} \rightarrow A_{ADP}</td>
<td>kw_A / ACYTO</td>
</tr>
</tbody>
</table>
| A_{ADP} \rightarrow A_{ADD}, i = 0...L-1 | \( k_m / L \times ACYTO \)

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doi:10.1371/journal.pcbi.1004009.t001
presence of par2, plasmids generally reside within the nucleoid region, while in its absence they tend to become somewhat more polar localized, although they can still sample the entire cell volume on long enough timescales [23]. Consistently we still find many par’ plasmids located within the nucleoid region (S3B Fig.). Restricting the mobility analysis to par’ plasmids within the nucleoid region did not alter the resulting MSD curves significantly (S3B Fig.). We conclude that the presence of par2 can increase plasmid mobility in the nucleoid region, which is inconsistent with a diffusion/immobilization mechanism. We emphasize that this conclusion can be made irrespective of the underlying (par’) plasmid transport processes, which we now describe in more detail.

It has been reported that chromosomal loci and RNA-protein particles exhibit subdiffusive, rather than diffusive, behavior in the cytoplasm [24,25]. Therefore it is possible that plasmids without a segregation mechanism could also exhibit subdiffusive motion. Further analysis is required to fully distinguish subdiffusion from the additional effects of cellular confinement or glass-like properties of the bacterial cytoplasm [23,25]. Nevertheless such additional analysis is not required for the conclusions on par’ plasmid mobility relevant to this study, as we now explain.

Subdiffusion results in an expected MSD displacement of the form $\langle r^2(\tau) \rangle = 4Dt^z$, with $z<1$ and $D$ the apparent diffusion constant (in units of $\mu m^2/s$). We find that our MSD displacements on both short and long timescales are well described by subdiffusion with $z = 0.7$–0.8 and an apparent diffusion constant $D = 5$–$10 \times 10^{-4}$ $\mu m^2/s$ (Fig. 3C and Materials and Methods for details). This is consistent with other recent reports on par’ plasmid mobility [23,26]. Importantly the experimental MSD is lower on all observed timescales than a hypothetical particle that would perform free diffusion inside a cell with a diffusion constant $D_f = 10 \times 10^{-4}$ $\mu m^2/s$. This upper limit is already much lower than that needed to be consistent with the previously reported plasmid displacement data discussed above. We will further exploit this upper limit in our analysis below.

To further investigate the effect of par2 on plasmid positioning, we also studied rapid plasmid segregation events. We defined these as cases where two plasmid foci whose separation is initially $\leq 0.3 \mu m$, move within 20 $s$ at least another $0.8 \mu m$ apart (Fig. 3A, S3C). We also allowed for the two foci to be initially merged. Using these criteria, despite equally large data sets, we found 13 such events in par2+ and only one such case in par’. Furthermore, we only retrieved 2 further par2+ segregation events when we relaxed the criterion to separation within 60 $s$ instead of 20 $s$. This analysis shows that most segregation events occur rapidly. When we investigated the 26 plasmid trajectories involved they showed larger maximal MSDs compared to sets of 26 trajectories that were repeatedly randomly sampled from the whole par2+ dataset (p $< 10^{-6}$). This finding indicates that the par2 system can particularly enhance the mobility of plasmids when they are in close proximity. We then simulated 300 plasmid duplication events with our diffusion/immobilization model to determine the magnitude of diffusion constant required to generate the experimentally observed segregation. Note that we used diffusion rather than subdiffusion here because we have already determined that par’ plasmid movement is slower on all observed timescales than free diffusion with a diffusion constant $D_f = 10 \times 10^{-4}$ $\mu m^2/s$. Hence, if the required diffusion constant is larger than $D_f$ then we have also ruled out a subdiffusion/immobilization model. We required that 5% (15 out of 300) of segregated distances within 20 $s$ were at least 0.3 $\mu m$ (a very conservative requirement, since the criterion was satisfied by 13 of our 15 experimental segregation events). This requirement necessitated a free plasmid diffusion constant on the order of $10^{-3}$ $\mu m^2/s$, about two orders of magnitude higher than our experimentally observed upper bound $D_f$ on the experimental par’ plasmid mobility. Hence, we conclude that the plasmids are generally too immobile for a diffusion/immobilization (or subdiffusion/immobilization) mechanism to explain these segregation events. Also the qualitative behaviour of segregation events in the diffusion/immobilization model appears different, since experimental segregation events (Fig. 3A, S3C) show more directionally biased motion, while the diffusion/immobilization model generates more sustained random, diffusive motion during segregation, prior to immobilization at equally spaced positions (Fig. 2B). Nevertheless, these segregation events were sufficiently rare not to significantly alter the overall MSD behaviour of the entire dataset shown in Fig. 3B. Thus the increased average mobility in the presence of par2+ cannot only be ascribed to these segregation events.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_A$</td>
<td>Nucleoid bound Par-A-ATP diffusion constant</td>
<td>$10^{-2}$ $\mu m^2/s$</td>
<td>Fitted, can be increased without loss of qualitative behaviour of system. Nevertheless, it is difficult to physically reconcile more mobile nucleoid-bound Par-A-ATP with the ability to immobilize a plasmid with a lower diffusion constant. Therefore we have assumed that Par-A-ATP diffuses 10× slower than the plasmid, ensuring that the assumption that Par-A-ATP can immobilize plasmids is physically justified.</td>
</tr>
<tr>
<td>$D_p$</td>
<td>Plasmid diffusion constant</td>
<td>$10^{-1}$ $\mu m^2/s$</td>
<td>A relatively high value is needed for compatibility with previous experiments [9]. An upper bound on the plasmid diffusion constant from experiments (Fig. 3C) turned out to be too slow for this model to fit our experimental observations.</td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>Par-A-ATP nucleoid binding</td>
<td>50 s$^{-1}$</td>
<td>Constrained by experiment [8].</td>
</tr>
<tr>
<td>$k_{AB}$</td>
<td>Par-A-ATP to plasmid binding</td>
<td>100 s$^{-1}$</td>
<td>Fitted, should be high enough to allow for plasmid immobilization.</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Plasmid bound Par-A-ATP hydrolysis (into Par-A-ADP) stimulated by ParB.</td>
<td>68.5 s$^{-1}$</td>
<td>Fitted together with $D_p$ and $k_W$ to ensure equal plasmid spacing.</td>
</tr>
<tr>
<td>$k_W$</td>
<td>(Cytoplasmic) Par-A-ADP to Par-A-ATP conversion</td>
<td>1/15 s$^{-1}$</td>
<td>Constrained by experiment [8], this value should be low enough to ensure that cytoplasmic Par diffusion can generate a uniform cytoplasmic Par-A-ATP and Par-A-ADP concentration.</td>
</tr>
</tbody>
</table>

Table 2. Parameter values used in the diffusion/immobilization model.
It is possible that the tetO-TetR-mCherry labeling system caused reduced plasmid mobility as compared to unlabelled plasmids. However, as we used the same labeling method for both par2+ and par− cases, our above conclusions on relative mobility are unaffected. Moreover, our tetO-TetR-mCherry labeled plasmids still exhibited rapid segregation events (such as in Fig. 3A), underscoring the ability of par2 to overcome low plasmid mobility. Overall, we find that diffusion/immobilization cannot explain our data on par2+ versus par− plasmid mobility, as well as on rapid par2+ plasmid segregation.

ParA structures competing to direct plasmid motion can space plasmids equally over the nucleoid

Given the shortcomings of the diffusion/immobilization model, we next tested models based on directed motion, allowing more rapid directed rather than unbiased diffusive plasmid movement. More specifically, we tested models based on the formation of competing ParA polymers, with ParB-parC-stimulated ParA-ATP hydrolysis directing plasmid movement. By modulating the length of these polymers, we thereby tested the robustness of directed motion models to generate equal plasmid positioning.

We again used a Gillespie algorithm to simulate ParA dynamics on the nucleoid (see Fig. 4A, Materials and Methods and Tables 3,4 for details). The nucleoid was represented as a rectangular lattice (dx = 5 nm in both dimensions), with a much shorter width (30 nm) than length (several μm). Similar reactions as in the diffusion/immobilization model described the cytoplasmic dynamics of ParA-ADP and ParA-ATP. Nucleoid-associated ParA-ATP could also still diffuse across the nucleoid in a mobile state in all four directions to neighbouring sites with diffusion constant \( D_A \). However, two of these molecules at sites neighboring each other along the long nucleoid axis could interact to form a ParA polymer of two subunits, with reaction parameter \( k_p \). Further ParA-ATP polymerization could occur by attachment of mobile ParA-ATP, located at a site immediately next to the tip of an existing ParA polymer, but only along the long axis. ParA-ATP polymers were assumed to be immobile. A ParA-ATP polymeric subunit could depolymerize spontaneously with reaction parameter \( k_d \), i.e. be converted into a mobile ParA structure competing to direct plasmid movement. By modulating the length of these polymers, we thereby tested the robustness of directed motion models to generate equal plasmid positioning.

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It is possible that the tetO-TetR-mCherry labeling system caused reduced plasmid mobility as compared to unlabelled plasmids. However, as we used the same labeling method for both par2+ and par− cases, our above conclusions on relative mobility are unaffected. Moreover, our tetO-TetR-mCherry labeled plasmids still exhibited rapid segregation events (such as in Fig. 3A), underscoring the ability of par2 to overcome low plasmid mobility. Overall, we find that diffusion/immobilization cannot explain our data on par2+ versus par− plasmid mobility, as well as on rapid par2+ plasmid segregation.

ParA structures competing to direct plasmid motion can space plasmids equally over the nucleoid.
along the long axis of that ParA-ATP subunit, coinciding with conversion of that ParA-ATP into cytoplasmic ParA-ADP. For wild-type simulations, any plasmid in the system formed a hard wall to mobile ParA-ATP diffusion so that diffusing ParA-ATP molecules could not diffuse past a plasmid.

We first adjusted the ParA-ATP polymerization rate to generate short filaments, of approximately 10 subunits in length (Table 4 for parameters). Simulations again faithfully reproduced the equal spacing of plasmids along simulated growing nucleoids with varying numbers of plasmids (np) in a simulated growing cell where a plasmid (red) is initially directed from a nucleoid edge to mid-cell by ParA (green) filament competition. After plasmid duplication, the system dynamically self-organizes to attain equal spacing.

ParA-GFP oscillations are not continuously required for equal plasmid spacing

Intriguingly, simulations of the directed motion model did not generally produce sustained spatiotemporal oscillations of ParA across the nucleoid (short polymers: Fig. 4B, long polymers: S4B Fig.). A lack of sustained oscillations would therefore appear to be a common feature of models where competitive ParA structures generate equal plasmid spacing. This absence was unexpected, as prior experimental work had emphasized the oscillatory aspect of the ParA dynamics [12–14]. To experimentally test this key model prediction in an unbiased fashion, we experimentally measured the degree of ParA asymmetry in the par2 system in a large dataset (n = 134) of snapshots of ParA-GFP across the nucleoid. We examined only cases with a single plasmid (tetO-TetR-mCherry focus, where sustained oscillations should be easiest to infer. The ParA-GFP fluorescence signal from pole to plasmid position was summed and divided by the respective pole-to-plasmid distance. This generated two ParA-GFP fluorescence densities IL and IR for either side extending to the two cell poles. This allowed us to compute the normalized asymmetry measure |IL−IR|/|IL+IR| [27] for ParA (see Materials and Methods for details). Asymmetric ParA-GFP distributions, arising for example from oscillations, where for example IL=0, IR=1, will give asymmetry values closer to one, whereas symmetric ParA-GFP distributions, where IL=IR, will give values closer to zero. Note that the ParA-GFP exposure time used here was 1.5 s; clearly, we cannot measure asymmetries that occur on a timescale faster than this exposure time. However, the timescales of the plasmid and ParA-GFP dynamics are on the order of tens of seconds or longer and it is therefore unlikely
that any significant asymmetry is being missed by our measurements.

When we examined our whole distribution of cells exhibiting single plasmid tetO-TetR-mCherry foci, we found that the degree of ParA-GFP asymmetry ([Fig. 5A,B]) was low in comparison with the well-established MinD spatiotemporal oscillator [27]. Furthermore, the ParA-GFP asymmetry did not correlate with cell length (S5A Fig., $R^2 = 0.08$), unlike the case of MinD-YFP [27]. We also compared the ParA-GFP asymmetry to the Hoechst signal. This DNA stain labels the nucleoid itself, which is relatively uniform along the long cell axis [28–30]. Here, any asymmetry is not expected to depend on the plasmid foci positions. The Hoechst asymmetry distribution was indeed concentrated around relatively small values, but was apparently measurable within our approach (Fig. 5B, S5B). Importantly, we found that the ParA-GFP asymmetry measure had a similarly low value as for the Hoechst case ([Fig. 5B, S5B, no significant difference, Kolmogorov-Smirnov test]), and that for both the asymmetry is uncorrelated to the plasmid focus position (S5C Fig.). We therefore conclude that for a single plasmid focus, ParA-GFP typically resides on both sides of a plasmid, with relatively little asymmetry or oscillation, as predicted by the directed motion model, irrespective of a weak (Fig. 5B) or strong (S5B Fig.) degree of polymerization.

Previous analyses had focused on plasmids migrating in the wake of retracting ParA-GFP structures [9]. Such events can transiently give rise to relatively high ParA-GFP asymmetries (see, for example, Fig. 3A, 5C). Accordingly, we conclude that ParA asymmetry or oscillations are not continuously required for parB mediated plasmid positioning.Transient asymmetry, including oscillations, instead likely arises from the dynamics needed to bring about equal plasmid spacing following a spatial perturbation or plasmid duplication event (Fig. 5C). Once the ParA distribution has returned to being relatively symmetric, this coincides with an equally spaced plasmid configuration (Fig. 5C). Such dynamics can be seen in our model simulations (Figs. 4B, S4B): asymmetric during plasmid segregation events, but relatively symmetric otherwise. This analysis can therefore accommodate both our findings of a relatively symmetric ParA distribution with previous reports emphasizing asymmetry and oscillations. Overall, our finding of predominantly symmetric, non-oscillatory ParA dynamics may help to reconcile similar findings for ParA in other plasmid partitioning systems, such as for plasmid P1 [15,16].

### ParA-GFP forms structures within the nucleoid region

One important feature to achieve equal plasmid spacing is that the ParA-ATP should be organized in a 1d-like structure along the
Table 4. Parameter values used in the directed motion models.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_A$</td>
<td>Nucleoid bound ParA-ATP diffusion constant</td>
<td>$1 , \mu m^2/s$</td>
<td>Constrained by experiment [18], value can be varied by several orders of magnitude without loss of qualitative behaviour of system. Note that this form of ParA-ATP does not have an effect on the mobility of plasmids, since only polymeric ParA-ATP, immobile due to the interaction with the nucleoid, can direct the motion of a plasmid.</td>
</tr>
<tr>
<td>$D_p$</td>
<td>Plasmid diffusion constant</td>
<td>$3 \times 10^{-4} , \mu m^2/s$</td>
<td>Constrained by experiment (Fig. 3C).</td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>ParA-ATP nucleoid binding</td>
<td>$50 , s^{-1}$</td>
<td>Constrained by experiment [8].</td>
</tr>
<tr>
<td>$k_a$</td>
<td>Plasmid bound polymeric ParA-ATP hydrolysis (into ParA-ADP) stimulated by ParB.</td>
<td>$68.5 , s^{-1}$</td>
<td>Chosen to be the same as $k_a$ in diffusion/immobilization model for consistency; constrained by $k_a \gg k_{on}$ which ensures that all the ParA-ATP at the location of a plasmid is converted into cytoplasmic ParA-ADP before the plasmid moves to a neighboring site. Value can be varied within a wide range without loss of qualitative behaviour of system.</td>
</tr>
<tr>
<td>$k_{mb}$</td>
<td>Plasmid bound mobile ParA-ATP hydrolysis (into ParA-ADP) stimulated by ParB.</td>
<td>$40 , s^{-1}$</td>
<td>Fitted, value can be varied within a wide range without loss of qualitative behaviour of system. Setting this rate too high depletes ParA-ATP locally around a plasmid, which inhibits directed plasmid motion events.</td>
</tr>
<tr>
<td>$k_W$</td>
<td>(Cytoplasmic) ParA-ATP to ParA-ATP conversion</td>
<td>$1/15 , s^{-1}$</td>
<td>Constrained by experiment [8], this value should be low enough to ensure that cytoplasmic ParA diffusion can generate a uniform cytoplasmic ParA-ATP and ParA-ADP concentration.</td>
</tr>
<tr>
<td>$k_mn$</td>
<td>Plasmid directed motion rate (in presence of one neighboring plasmid)</td>
<td>$0.8 , s^{-1}$</td>
<td>Constrained by experiment (Fig. 2A). If interpreted as biased plasmid diffusion along the polymer (burnt-bridge mechanism [4]), this would result effectively in a maximal plasmid diffusion constant of $1 \times 10^{-4} , \mu m^2/s$ (short) and $1.2 \times 10^{-4} , \mu m^2/s$ (long). These values are consistent with the free diffusion constant $D_p$ (see above), since the interaction with immobile ParA-ATP polymers could lower the plasmid mobility.</td>
</tr>
<tr>
<td>$k_p$</td>
<td>Polymerization: mobile ParA-ATP to polymeric ParA-ATP conversion</td>
<td>$800 , s^{-1}$ (short), $10^5 , s^{-1}$ (long)</td>
<td>Fitted together with $k_{on}$ and $k_W$ to ensure equal plasmid spacing. $k_p$ and $k_{dp}$, together with the total ParA-ATP concentration determine the extent of ParA-ATP polymerization.</td>
</tr>
<tr>
<td>$k_{dp}$</td>
<td>Spontaneous depolymerization: ParA-ATP to mobile ParA-ADP conversion</td>
<td>$10 , s^{-1}$ (short), $10^{-4} , s^{-1}$ (long)</td>
<td>Fitted. See notes on $k_p$ parameter above.</td>
</tr>
<tr>
<td>$S$</td>
<td>Short axis length of the nucleoid region where nucleoid bound ParA-ATP can polymerize.</td>
<td>30 nm (short), 25 nm (long)</td>
<td>Fitted, values should be small compared to the long nucleoid axis length to ensure that segregation occurs along the long nucleoid axis.</td>
</tr>
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</table>

**Perturbed nucleoid simulations**

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Value</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>$k_{mb}$</td>
<td>Plasmid-bound mobile ParA-ATP hydrolysis (into ParA-ADP) stimulated by ParB.</td>
<td>$4 , s^{-1}$</td>
<td>Fitted, value is chosen to simulate the effect of a disordered nucleoid structure, allowing mobile ParA-ATP to diffuse past plasmids.</td>
</tr>
<tr>
<td>$S$</td>
<td>Short axis length of the nucleoid region where nucleoid bound ParA-ATP can polymerize.</td>
<td>30 nm (short), 10 nm (long)</td>
<td>Fitted, values are chosen to ensure a sufficient amount of mobile ParA-ATP.</td>
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nucleoid as concluded above. However, it is unclear why ParA-ATP on either side of a plasmid would align in a coherent 1d-like structure with their ends coinciding with a plasmid. One potential explanation for this 1d-like behavior is that the ParA-ATP structures are sensitive to the overall nucleoid architecture. To test these features, we examined the localization of ParA-GFP and Hoechst signal simultaneously using optical sectioning in WT cells ($n = 678$) without par2-carrying plasmids to prevent dynamic ParA-GFP structure disassembly. ParA-GFP intensity correlated well with the DNA stain (Fig. 6A,B, S6A). Pearson’s correlation coefficient $r_p = 0.81$, indicating that ParA-GFP localization was indeed dependent on the underlying nucleoid. Importantly, ParA-GFP overlaid more with Hoechst than the reverse (Fig. 6C), indicating that ParA forms structures within the nucleoid region rather than uniformly covering the nucleoid. Although the resolution of our techniques does not allow identification of potential individual ParA polymers, in many cases we did observe extended 1d-like ParA-GFP structures on the nucleoid (Fig. 6B, S6A). Care must be taken in interpreting fluorescent localization studies due to potential artifacts, for example GFP-induced polymerization [31]. However, wild-type plasmid loss rates and plasmid foci positioning in cells expressing ParA-GFP argue against localization or polymerization artifacts in our case [9,12].

**Equal plasmid spacing is compromised in cells with a perturbed nucleoid**

We reasoned that if ParA structures are reliant on the nucleoid morphology for their organization, then mutants/treatments that perturb the overall nucleoid structure should also exhibit alterations in ParA localization and therefore plasmid focus positioning (Fig. 7A). We measured plasmid focus positioning in mukF, mukF and malD mutant strains, as well as in cells treated with the DNA gyrase inhibitor nalidixic acid (Nal), all of which exhibit defects in nucleoid organization [32–34]. Nucleoid length...
distributions were altered in all of these cases (S7A Fig.) and, consistent with our hypothesis, there was in each case a similar deterioration in the fidelity of plasmid focus positioning (np = 1,2 in Fig. 7B, S7B,C, np = 3,4 in S7B,C,D Fig.) towards a random distribution (S7E Fig.). This deterioration may not have been large enough to detect in stability assays [35,36]. Similarly, in E. coli mukB mutants, perturbed plasmid positioning without compromising plasmid stability has also been observed, although for the segregation mechanism mediated by ParM [37]. The deteriorations in plasmid positioning could have resulted from other effects.

Fig. 5. As predicted by the directed motion model, ParA-GFP distribution is relatively symmetric. (A) Localization of plasmids and summed Z-stack of ParA-GFP distributions in a field of cells. Scale bar: 1 μm; plasmid: pSR233 (mini-R1, par2+, P<sub>lac</sub>::parA::eGFP, tetO120) in E. coli cells harboring pSR124 (P<sub>BAD</sub>::tetR::mCherry). (B) ParA-GFP (n = 134) asymmetry measure |IL-IR|/|IL+IR| using fluorescence densities IL, IR on left, right sides of a plasmid focus along long cell axis (see Materials and Methods). Comparison shown to the prediction of directed motion model with short polymers, Hoechst (n = 134) and MinD–YFP case [27]. Box plots represent minimal, first quartile, median, third quartile and maximal values of asymmetries in all cases. (C) Time lapses showing localization of par2+ pSR236 (mini-R1, parC1+, parA+, parB+, parC2+, P<sub>lac</sub>::parA::eGFP, tetO120) plasmids in E. coli cells harboring pSR124 (P<sub>BAD</sub>::tetR::mCherry).

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such as an induction of the SOS response in Nal-treated cells. However, the similarity of the altered plasmid positioning in all four cases instead suggests a common positioning defect based on nucleoid perturbation. This deterioration could also be due to an altered plasmid structure. However, at least for the case of matP we do not favor this hypothesis, due to the absence of MatP target sites (matS) on our test-plasmid.

To provide evidence that the above deterioration in plasmid positioning arose from an altered ParA distribution, we systematically examined localization of ParA-GFP and Hoechst stain simultaneously in Nal-treated cells ($n = 862$), which had the largest visible perturbations. We were able to quantify (Fig. 7C, S6B, $p < 10^{-149}$, see Materials and Methods) perturbations in nucleoid structure that were detectable by eye (Fig. 6A, S6A). Moreover, visual inspection showed that the ParA-GFP distribution followed the nucleoid structure less closely than in the WT (Fig 6A, S6A). This finding was quantitatively confirmed by a correlation coefficient of $r_P = 0.68$, decreased from its WT value of 0.81 ($p < 10^{-34}$), and also by a decrease in the ParA-GFP overlap coefficient (Fig. 7C). Altogether, these findings support our hypothesis that the nucleoid provides a template for 1d-like ParA-ATP structure formation, which is partially compromised when the nucleoid structure is perturbed.

To reproduce this behavior in the directed motion model, we assumed that mobile DNA-bound ParA-ATP could now diffuse past a plasmid (see Materials and Methods for details). This could be due to the disordered nucleoid structure resulting in a deteriorated ParA-ATP structure organization, thereby allowing ParA-ATP to spatially bypass ParB-parC complexes and compromise the ParA concentration differences between either side of a plasmid. The directed motion model with a weak (Fig. 7D, S7F Fig.) or strong (S7F Fig.) degree of polymerization could then reproduce the observed plasmid focus distributions (Fig. 7B).

**Discussion**

Stable DNA inheritance is important for the viability of essentially all organisms. In bacteria, the parABC genes have a major role in this process for plasmid DNA [1]. In this study, we have investigated how *E. coli* utilizes the par2 partitioning system from plasmid pB171. We have for the first time provided a robust mechanistic explanation for how plasmids are equally spaced over the nucleoid, a process vital for the fidelity of low copy number plasmid inheritance. We propose that competing ParA structures function to direct plasmid movement over the nucleoid to equally spaced positions. This mechanism is likely relevant to other parABC systems that move and position sub-cellular objects.

It has previously been proposed that plasmid positioning is controlled by concentration gradients of ParA-ATP over the nucleoid, caused by plasmid-associated ParB-parC complexes mediating ParA-ATP hydrolysis [8,16–20]. In this so-called diffusion-ratchet mechanism [8,17,18,20], it has remained unclear whether such a mechanism could actually mediate equal plasmid spacing, and if so, which specific properties of the system were key. In particular, it was left unclear how ParA actually influenced plasmid movement [8,17,18], e.g. through immobilizing plasmids or actively directing their motion through a chemophoresis force [19,20]. Furthermore, although the diffusion-ratchet mechanism did not strictly preclude some degree of ParA polymerization, its
Fig. 7. Nucleoid morphology disruption causes aberrant plasmid focus positioning. (A) Fluorescence localization of ParB-GFP (green) and Hoechst DNA stain (blue) in mukE, mukF, matP mutants and wild-type cells treated with 50 μg/ml nalidixic acid (Nal). Scale bar: 1 μm; plasmid: pFS21 (mini-R1, parC1+, parA+, parB::sfGFP, parC2+). (B) Histograms of plasmid foci positions (n = 1,2) for mutants/treatments described in (A) relative to nucleoid size. According to Kolmogorov-Smirnov tests, all distributions are broader than WT (Fig. 1C) with p<10^{-2} except Nal n_p = 1: p<0.05. (C) Manders overlap coefficients (error bars: standard error of the mean) of ParA-GFP comparing WT (n = 678) and Nal-treated cells (n = 862). Consistent with a decrease in the Pearson’s correlation coefficient r_p (p<10^{-38}), ParA-GFP overlaps less with Hoechst in Nal-treated cells as compared to WT (p-values ranging from 10^{-14} to 10^{-25}). (D) Time-averaged plasmid position distributions for directed motion model with short polymers obtained as in Fig. 2C from 124 independent simulations. Here, mobile DNA-bound ParA-ATP was now able to diffuse past a plasmid.

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through the action of plasmid-associated ParB-movement. In particular, we assumed that ParA-ATP could form a certain extent cytoplasmically, and subsequently binds and mobility in the presence of polymer models could naturally explain enhanced plasmid concentration at a particular location. We found that ParA with the degree of polymerization influenced by the overall ParA ParA-ATP could bind to the nucleoid, diffuse and then relatively large intracellular components, given the glass-like earlier measurements [23,26,38] and is likely a general feature for duplication events. This intrinsically low mobility agrees with experimentally-observed rapid plasmid segregation following overall free plasmid mobility was too low to allow the higher in the presence rather than the absence of predictions did not verify: in particular, plasmid mobility was elevated when we tested this model experimentally, its polymerize at all, but nevertheless forms dense structures on the nucleoid with many ParA-ATP contacting a plasmid at any given time. In this variant, biased diffusion through an analog of a “burnt-bridge” mechanism is still possible. Another possibility is that ParA-ATP does not polymerize at all, but nevertheless forms dense structures on the nucleoid DNA to which ParA-ATP dimers are bound [21]. Moreover, plasmid diffusion seems not always required for directed plasmid movement. Brownian dynamics simulations based on ParB-parC-mediated disassembling ParA polymer bundles can both tether and pull plasmids simultaneously without the need for plasmid diffusion [39]. We propose that distinct underlying translocation mechanisms, as exemplified above, could be responsible for directed motion in different parABC systems and yet still attain similar equal plasmid spacing. For our models to generate equal plasmid spacing, ParA should be organized into a 1d-like configuration along the nucleoid. If ParA were not organized in this way, it would be possible for ParA to diffuse around the sides of a plasmid without encountering the hydrolyzing effect of the ParB-parC complex. This would equalize the ParA concentrations on both sides even in the case of asymmetrically placed plasmids, leading to failure of the equal spacing mechanism. Potentially such ParA structures could consist of long ParA polymer bundles, or an extended region containing short ParA polymers or dimers. Importantly, in this work, we have provided experimental evidence for such ParA structure formation within the nucleoid region. Interestingly, it has been reported that the E. coli chromosome adopts a helical shape [29,30]. Potentially the ParA structures could be preferentially located within a “valley” in this configuration, thereby naturally generating a 1d-like appearance, even for dimers or short polymers. Consistent with these concepts, we found experimentally that plasmid positioning is compromised in nucleoid perturbed strains. ParA structures could also provide a high enough ParA concentration to ensure plasmid tethering and directed plasmid motion, whilst preventing plasmids from diffusing away from the nucleoid, a process which would compromise regular positioning. Further investigation of the exact involvement of the nucleoid in intracellular cargo positioning is therefore an important future goal.
Materials and Methods

Diffusion/immobilization model

On the one dimensional lattice with sizes of \( dx = 5 \) nm, sites are numbered \( 0...L-1 \). Reactants are \( A_m \) ParA-ATP at site \( i \) with number \( A_m[i] \) (\( \geq 0 \)), \( P_{i,j} \) plasmids with \( j \) ParA-ATP bound to it at site \( i \) with number \( P_{i,j}[i] \) (\( \geq 0 \)). \( A_{\text{ADP}} \) cytoplasmic ParA-ADP with number \( A_{\text{ADP}} \) (\( \geq 0 \)), \( A_{\text{ATP}} \) cytoplasmic ParA-ATP with number \( A_{\text{ATP}} \) (\( \geq 0 \)). The reactions and corresponding propensities \( p_i \) are described in Table 1. Parameter values used are listed in Table 2.

We varied the exact number of ParA-ATP molecules forming a complex that are required to completely immobilize the plasmid and this variation does not alter the qualitative behavior of the system. Introduction of a low spontaneous ParA-ATP hydrolysis parameter \( k_{\text{off}} \) also does not alter the behaviour of the system. We do not keep track of the spatial positions of ParA-ADP and ParA-ATP in the cytoplasm. Instead we merely keep track of their number.

The ParA concentration is assumed to be constant throughout the cell cycle, consistent with the total ParA-GFP fluorescence as a function of cell volume when expressed from an inducible promoter (S1C Fig.). In accordance with estimates for average ParA copy numbers obtained by semi-quantitative Western blots (S1B Fig.), the ParA concentration is assumed to be 2400 ParA (dimers) per \( \mu m \) of nucleoid. Simulations start at time \( t = 0 \) and run until time \( t \), updated according to the Gillespie algorithm, exceeds a pre-defined time \( T \). To simulate nucleoid growth during the cell cycle the nucleoid lattice is extended by two sites of size \( dx \) (not containing any ParA or plasmids), at one randomly chosen position along the nucleoid length. Such a growth event occurs at regular time intervals. Reaction propensities are then updated in accordance with the new state.

In Fig. 2B the nucleoid grows from 1.5 \( \mu m \) to 3 \( \mu m \) in \( T = 40 \) min, reflecting one cell cycle. Initially a quarter of the total ParA in the system is in the cytoplasmic ParA-ADP form, 11 \( T = 40 \) min, reflecting one cell cycle. Initially a quarter of the total ParA in the system is in the cytoplasmic ParA-ADP form, 11

Plasmids and strains

The ParA-GFP fusion and tetO-TetR-mCherry plasmid labeling system were described previously [9,12]. To obtain the functional ParB-GFP fusion, the \( \text{parB} \) gene in the \( \text{par2} \) locus was replaced by \( \text{parB::gfp} \) and inserted in a mini-R1 test-plasmid. See S2 Text for more details on the strains and plasmids construction, semi-quantitative ParA western blotting and supplemental figure data analysis.

Epifluorescence microscopy

E. coli strains carrying plasmids of interest (see Table S1, S2 in S2 Text for details on strains and plasmids) were grown to stationary phase while being shaken at 37°C in LB medium supplemented with appropriate antibiotics (30 or 50 \( \mu g/ml \) ampicillin, 25 or 50 \( \mu g/ml \) kanamycin, 15 \( \mu g/ml \) chloramphenicol), with the exception of the \( \text{muk} \) strains, which were grown at 24°C. Cultures were diluted to an \( \text{OD}_{590} \) of 0.025 in antibiotic-free M9 minimal medium containing supplements (0.2% casamino acids, 0.2% glycerol, 1 \( \mu g/ml \) thiamine, 1 mM MgSO\(_4\), 0.1 mM CaCl\(_2\)). Inoculated cultures were incubated until an \( \text{OD}_{590} \) of \( \approx 0.2 \) was reached, typically taking 3 h.

When nalidixic acid was used to condense the nucleoids, the antibiotic was added to a growing culture at a final concentration of 50 \( \mu g/ml \) two hours before imaging. Where appropriate, culture samples were mixed with Hoechst 33342 (Invitrogen) at a final concentration of 50 \( \mu g/ml \) for DNA staining immediately before microscopy.

For imaging, cells were immobilized on 1.5% agarose-M9 pads mounted on microscopy slides using Gene Frames (Thermo Scientific). All microscopy experiments, unless specified otherwise (see below), were carried out using an Olympus IX71 inverted microscope with a CoolSNAP HQ EMCCD digital camera.
thereafter. Expression of TetR-mCherry was not induced, as samples were treated with Hoechst stain and imaged immediately.

Samples were treated with IPTG to the culture medium one hour before microscopy. A 31 image Z-stack with 0.1 mm section widths was taken for all projections (exposure times Phase Contrast (GFP channel): 0.05 s, ParA-GFP: 1.5 s, ParA::ParB::sfGFP, TetO120) was induced by adding 10 µM IPTG to the culture medium one hour before microscopy. A 31 image Z-stack with 0.1 mm section widths was taken for all projections (exposure times Phase Contrast: 0.1 s; TetR-mCherry: 1.5 s, ParA-GFP: 1.5 s, ParA::ParB::sfGFP, TetO120).

Image stacks were subsequently deconvolved using SoftWoRx v.5.5.0 with the following parameters: 10 iterations, medium noise reduction, conservative method.

### Measuring asymmetry in ParA-GFP distributions using optical sectioning

Expression of ParA-GFP from plasmid pSR236 (mini-R1, parC1, parC2, P_tac::parA::eGFP, tetO120) in E. coli strain KG22 cultures for 15 minutes, followed by catabolite repression with 1% glucose for 10 minutes. In strains harbouring pSR236, expression of TetR-mCherry provided from the pSR124 vector (see [40] for the original method). TetR-mCherry expression was induced by adding L-arabinose to a final concentration of 0.02% to growing cultures for 15 minutes, followed by catabolite repression with 1% glucose for 10 minutes. In strains harbouring pSR236, expression of ParA-GFP was induced by the addition of 10 µM IPTG inducer 2 h before microscopy. Time-lapse image series were acquired for different total durations/time intervals: 1 min/4 s or 15 min/30 s for pMH82tetO120 (exposure times phase contrast: 0.1 s; TetR-mCherry: 1.5 s) and 1 min/5 s or 15 min/20 s for pSR236 respectively (exposure times phase contrast: 0.1 s; TetR-mCherry: 1.5 s; ParA-GFP: 1 s). The maximum rate of image acquisition possible with our imaging system was every 4 s and 5 s (without and with ParA-GFP channel) for pMH82tetO120 and pSR236 respectively. Sample focus was maintained in the mid-cell plane throughout the experiment using the UltimateFocus system (Applied Precision) sampling and refreshing before the acquisition of each individual frame.

### Plasmid focus positioning microscopy

E. coli strains KG22, FSI (KG22::AmprP), SF2 (KG22::mukE::kan) or FS3 (KG22::mukE::kan) harbouring pFS21 (mini-R1, parC1, parA1, parB1::sfGFP, parC21) were grown to an OD600 of 0.3. Samples were treated with Hoechst stain and imaged immediately in the mid-cell plane (exposure times ParB-GFP: 1 s, Hoechst: 0.5 s). Images of muk strains were acquired using a Zeiss Axiovert 200 M inverted epifluorescence microscope with a Zeiss Plan-Neofluar 100X/1.3 NA oil objective in a temperature-controlled room at 22°C. The microscope was controlled using the MetaMorph software version 7.7.5.0 ( Molecular Devices, Inc.). Cells were illuminated using a Lambda LS xenon-arc lamp and images acquired using a CoolSnap HQ2 EMCCD digital camera (Photometrics, pixel size = 0.0625 µm). Filter set specifics are given in Table S3. Other strains were imaged using both the Olympus IX71 and Zeiss Axiovert 200 M systems described above.

### Plasmid foci mobility determination

Using the MATLAB-based software suit MicrobeTracker (MT) [27], we determined E. coli cell outlines from phase contrast (PC) images, as well as the distribution of tetO-TetR-mCherry-labeled plasmids along the long axis of cells. The cell outlines were used together with the MATLAB tools spotFinderZ and spotFinderM [27] to determine tetO-TetR-mCherry foci positions in par time-lapses of 1 min (short) or 15 min (long) in duration with images taken at intervals of 4 s or 30 s respectively. The linear tetO-TetR-mCherry distribution was used to control the peak detection method for false positives/negatives. For the short time-lapses we analysed cells with one or more foci, although all our results were unchanged if analysis was restricted to one focus cells to prevent potential foci labelling errors. For the long time-lapses, we only analysed cells exhibiting one focus. This was due to difficulties in distinguishing between multiple foci due to merging/splitting events, out of focus plane movement and photobleaching when acquiring images using a time interval of 30 s. These effects could have resulted in biases in the analysis due to labelling errors. We were unable to lower the time interval and simultaneously image for long time periods due to TetR-mCherry photobleaching.

At every time point the two-dimensional squared foci displacements $r^2(t)$ after time lag $\tau$ were determined. All measured displacements for the same time lag were then averaged together to obtain Mean Square Displacements (MSD) $\langle r^2(t)\rangle$ with time lags from 4 s to 15 min (Fig. 3B,C, S3B). The measured plasmid displacement $r(t)$ can report the true plasmid displacement $r_p(t)$ at a resolution no greater than our measurement error, which can be up to 0.1 µm due to microscope drift. Our measurements are also limited by a finite pixel size of 0.066 µm. We therefore have: $r(t) = r_p(t) + \epsilon$, where $\epsilon$ is the error due to both of the above effects. Squaring and averaging over many plasmid trajectories results in an MSD: $\langle r^2(t)\rangle = \langle r_p^2(t)\rangle + \langle \epsilon^2 \rangle + \langle 2r_p \epsilon(t) \rangle$. The last term vanishes due to averaging; but the second term remains and generates a small time independent value for $\tau > 0$. Even at short time-scales of up to a minute, the MSD has a nonlinear shape, as has been reported before [26]. This is fully consistent with subdiffusive motion on these timescales. We thus expect the experimentally observed planar MSD for free particle subdiffusion in three dimensions to have the form: $\langle r^2(t)\rangle = 4Dt^2 + \beta$. We
carefully measured the par MSD up to 1 min with short time intervals between measurements (Fig. 3B). We performed a nonlinear least squares fit (weighted by the standard error of the mean (SEM): 1/SEM(t)) for \( \langle r^2(t) \rangle = 4Dt^2 + \beta \) resulting in the values \( \alpha = 0.78 \pm 0.04 \), \( D = 6.8 \pm 1.2 \times 10^{-4} \text{ mm}^2 \text{s}^{-1} \), \( \beta = 6 \pm 1 \times 10^{-3} \text{ mm}^2 \). The asymmetry distribution (Fig. 3B) therefore reflects the experimental MinD-YFP asymmetry with respect to mid-cell in large cells [27]. In this way, we can directly compare the asymmetry present in the ParA-GFP and Hoechst signal distributions with that induced by the spatiotemporal oscillations of MinD-YFP. We also generated asymmetry measures using our directed motion model.

### Three dimensional nucleoid and ParA structure analysis

To compare the extent of overlay and 3D structure of Hoechst (nucleoid DNA) stain and ParA-GFP, we first had to align the Z-stack pairs in an unbiased manner. To achieve this, one phase contrast (PC) image (at mid z height) of the Hoechst signal sections was aligned with one GFP section PC image (at the same z position) using the TurboReg ImageJ plugin (option: translation) [41], after cropping both PC images to match the output size of the deconvoluted Z-stacks. Using the same translation as for the Hoechst PC image, Hoechst Z-stacks were then translated in ImageJ to align them with the ParA-GFP Z-stacks.

We determined cell outlines in MT as described above using the PC image acquired with the GFP channel and excluded cells that did not show visible ParA-GFP and Hoechst stain simultaneously. We then computed the linear distributions (for every z height) along the long cell axis for the deconvoluted Hoechst and ParA-GFP Z-stacks. We next determined for the ParA-GFP and Hoechst signals separately in every cell the maximal intensity value in the whole cell (Imax(cell)) and the maximal values at every z height (Imax(z)). To find the 9 z planes from the Z-stacks (dz = 0.1 mm) that are in-focus for each cell in an automated fashion, we summed Imax(z) over 9 consecutive z positions including a given starting plane and determined the starting plane that gave the largest associated summed value. This starting plane and its 8 consecutive planes formed the in-focus plane set.

We verified that this method generated the right focus planes by inspecting the chosen planes visually for several cells. This method circumvents the problem of different focus planes for cells on the same image stack as well as alignment inaccuracies in the z direction between ParA-GFP and Hoechst signals which are difficult to control for manually.

Visual comparison of the nucleoid shape between WT and nalidixic acid (Nal) treated cells (Fig. 6A,B, S6A) revealed clear...
differences. In Nal-treated cells, the nucleoid signals, where present inside a cell, were more uniform along the long cell axis than in the WT (S6A,B Fig.). Shape differences were also visible in the raw Z-stacks suggesting they were not artefacts of the deconvolution method. To quantify these shape differences in an unbiased and systematic manner, we performed the following analysis (S6B Fig.).

We reasoned that a more uniform pattern would result in a profile along the long axis that resembled a first harmonic (first non-constant term of a Fourier expansion) between the nucleoid edges. Such a harmonic would not fit so well to a more spatially oscillating pattern that would arise, for example, from helical structures. Using the Hoechst stain \( I_{maxcell} \) and the \( I_{max}(x,z) \) arising from the 9 relevant focus planes we determined the half-maximum intensity locations along the long cell axis closest to the cell poles \( x_L \) and \( x_R \) at every \( z \) height. At every focus plane \( z \) height we could now define the ‘first harmonic’ function defined for \( x_L \leq x \leq x_R \):

\[
H(x,z) = \frac{I_{max}(z)}{I_{maxcell}} \left(1 + \frac{1}{2} \sin \left(\frac{\pi(x-x_L)}{x_R-x_L}\right)\right)
\]

For every \((x,z)\) we calculated the squared error \( SE(x,z) \) between the actual intensity value \( I(x,z) \) and \( H(x,z) \): \( SE(x,z) = [I(x,z) - H(x,z)]^2 \). Lastly we summed over the SEs at every \((x,z)\) and divided by the number of position points \([x,z]\) to obtain a single measure of deviation \( SE_{cell} \) in each cell that is independent of the number of data points (and thus nucleoid size) and expression level variation between cells (because of normalization to \( I_{maxcell} \)). We then performed a Wilcoxon rank sum test on the set of \( SE_{cell} \) comparing a population of WT cells with nucleoid-perturbed cells (\( n_{WT} = 678 \) and \( n_{Nal} = 862 \)). Nucleoid shapes in Nal-treated cells were indeed altered (\( p<10^{-149} \)). Note that this method did not detect a notable shape change in \( matP \) cells (\( n_{matP} = 579 \)), potentially due to our techniques not being sufficiently sensitive.

To quantitate the colocalization of ParA-GFP and Hoechst signal in each cell, we also calculated, for every cell, the Pearson’s correlation coefficient \( r_P \) using all the intensity values \( I_{ParA,GFP}(x,z) \) and \( I_{Hoechst}(x,z) \) [42].

To determine the fraction of ParA-GFP intensity signal that overlaps with Hoechst signal and vice versa we computed Manders overlap coefficients [42]. This method requires a choice of threshold \( T_{Manders} \) to distinguish between positions \((x,z)\) that are considered to contain or lack sufficient intensity signal. We therefore performed our analyses for the complete range of \( T_{Manders} \) (nWT = 678 and nNal = 862). Nucleoid shapes in Nal-treated cells were indeed altered (\( p<10^{-149} \)). Note that this method did not detect a notable shape change in \( matP \) cells (\( n_{matP} = 579 \)), potentially due to our techniques not being sufficiently sensitive.

Likewise the Manders overlap coefficient of Hoechst onto ParA-GFP is defined as:

\[
M_{Hoechst} = \frac{\sum_x \sum_z I_{Hoechst, colo}(x,z)}{\sum_x \sum_z I_{Hoechst}(x,z)},
\]

with

\[
I_{ParA-GFP, colo}(x,z) = \begin{cases} 
1_{ParA-GFP}(x,z) & \text{if } I_{Hoechst}(x,z) \geq T_{Manders} \\
0 & \text{otherwise}
\end{cases}
\]

Note that taking \( T_{Manders} = 0 \), will generate an overlap coefficient of one by construction. The normalization to \( I_{maxcell} \) in determining the colocalizing positions allows the overlap coefficients to be comparable between cells.

In a small fraction of cells the alignment procedure described above did not result in proper alignment. This is clearly reflected in the \( r_P \) values being considerably lower for these cases than for the cell population mean \( r_P \) value. However, without excluding these few, possibly false negatives, cases the population mean \( r_P \) value is still high (0.81 and 0.68 for WT and Nal-treated cells respectively), indicating that ParA-GFP and Hoechst signals generally correlate strongly at a population level. Poor alignment affects Manders overlap coefficients for the ParA-GFP and Hoechst signals on average equally and is not biased towards a particular strain/treatment. Therefore the observed misalignment of a small fraction of cells does not affect the qualitative conclusions that we state in this study.

Note that in \( matP \) cells, we did not observe any significant alteration in intensity correlation (\( r_P = 0.80 \) for \( matP \)), nor ParA-GFP overlap coefficient, as compared to the WT. This result was expected given that we could not detect any significant nucleoid structure alteration, as described above.

Supporting Information

S1 Fig par2 protein functionality and expression levels. (A) Plasmid loss-freency assay showing pFS21 stabilisation to wild-type levels by the recombinant par2 locus encoding parB::sfGFP, confirming functionality of the fluorescent fusion protein. Plasmids used are pRR200 (par2, red), pFS19 (par2+, black) and experimental vector pFS21 (parC1+, parA+, parB::sfGFP, parC2+, green), n = 2, error bars: standard error of the mean. (B) Representative section of semi-quantitative Western blot used for approximating ParA molecule numbers \( m \) in vivo. Cell lysate samples of strain KG22 carrying a mini-R1 plasmid lacking [pRR200] or containing par2 [pGE2] were compared to plasmid-free KG22 cell lysate mixed with known amounts of purified His6ParA. Standard curve generated from intensity measurements from this blot has \( R^2 = 0.965 \). Band intensities were measured and quantified using the Image-Quant TL 1D Gel Analysis Software, (n = 3). (C) Scatter plot of ParA-GFP total fluorescence signal in single WT cells as a function of cell volume, when expressed from an inducible promoter (\( P_{hsp} \)). The different color labels indicate the number of plasmid foci. Plasmids: pSR233 (mini-R1, par2+, P\( hsp ::parA::eGFP, tetO120 \)) and pSR124 (\( P_{BAD::tetR::mCherry} \)). (D) Scatter plot of Par-B-GFP total fluorescence signal in single WT cells as a function of cell volume, when expressed from its native promoter. Plasmid: pFS21 [parC1+, parA+, parB::sfGFP, parC2+]; color labeling as in (C). (PDF)
S2 Fig Plasmid foci are equally spaced over the nucleoid irrespective of nucleoid length or plasmid focus copy number. (A) Scatter plot of plasmid foci positions (blue, green, red, cyan) with respect to nucleoid edges (purple) and cell edges (black) for wild-type cells. Strains and plasmids used for S2 Fig. are as described in Fig. 1. (B) Histograms of plasmid foci positions shown in (A) relative to nucleoid length.

S3 Fig Diffusion/immobilization model can move and maintain plasmids at equally spaced positions. (A) Time-averaged plasmid position distributions for diffusion/immobilization model with \( n_p = 3,4 \) on a simulated nucleoid growing from 1.5 \( \mu \text{m} \) to 3 \( \mu \text{m} \) in 40 min without plasmid duplication. Plasmid distributions were obtained by sampling positions every 5 s in 36 independent simulations. (B) Plots as in Fig. 3B except with experimental \( par^- \) (red, green, blue) plasmid trajectories in which plasmid location is within a region of normalized Hoechst stain intensity I equal to or higher than the values indicated in the legend. The corresponding plasmid copy numbers (\( n_{par^+} = 763, n_{par^-} = 747, n_{par^-} = 592, n_{par^-} = 501 \)) indicate that a large fraction of \( par^- \) plasmids do indeed reside in the nucleoid region; error bars: standard error of the mean. (C) Plots of 13 segregation events of \( par^2 \) pSR236 (mini-R1, \( parC^1 \), \( parA \), \( parB \), \( parC^2 \), tetO120, \( PBAD::tetR::mCherry \)) plasmids in \( E. coli \) cells harboring pSR124 \( (PBAD::tetR::mCherry) \). Shown is the additionally segregated distance (colored lines) as a function of time, both with respect to and the position of each segregation event. A segregation event is defined as two foci that are initially \( \leq 0.3 \mu \text{m} \) apart and subsequently segregate \( \geq 0.8 \mu \text{m} \) further apart within 20 s. The horizontal line (black) indicates 0.8 \( \mu \text{m} \).

S4 Fig The directed motion model can equally space plasmids over the nucleoid, and is not critically dependent on the extent of ParA polymerization. (A) Time-averaged plasmid position distributions for directed motion model with short polymers with \( n_p = 3,4 \) plasmids on a simulated nucleoid growing from 1.5 \( \mu \text{m} \) to 3 \( \mu \text{m} \) in 40 min without plasmid duplication. Plasmid distributions were obtained by sampling positions every 5 s in 36 independent simulations. (B) Typical simulation kymograph of the directed motion model with long polymers. Long polymers extend from nucleoid ends in a growing cell, where plasmid (red) is initially directed from a nucleoid edge to mid-cell by ParA (green) filament competition. After plasmid duplication, the system dynamically self-organizes to reacquire equal plasmid spacing. (C) Time-averaged plasmid position distributions for directed motion model with long polymers with \( n_p = 1–4 \) plasmids. Simulated nucleoid growth and plasmid distributions obtained as in (A).

S5 Fig Hoechst DNA stain and ParA-GFP signal asymmetry are relatively low and uncorrelated to plasmid focus positioning. (A) Scatter plot of ParA-GFP asymmetry measure as a function of cell length (\( n = 134 \)). (B) ParA asymmetry prediction from the directed motion model with long polymers. Comparison shown to experimental ParA-GFP (\( n = 134 \)), Hoechst (\( n = 134 \)) and MinD-YFP distributions [7]. (C) Scatter plot of ParA-GFP and Hoechst asymmetry as a function of (a single) plasmid focus position relative to cell length.

S6 Fig Correlation between Hoechst and ParA-GFP distributions. (A) Normalized fluorescence intensity profiles along the long cell axis for 9 in focus z heights (\( dz = 0.1 \mu \text{m} \)) resulting from deconvolved Z-stacks in representative WT and Nal-treated strains. Many cases (representative examples shown) support the existence of linear ParA-GFP structures, although the inherent optical resolution of the imaging prohibits stronger conclusions about the presence or absence of narrow linear ParA-GFP filaments. For every cell having detectable Hoechst and ParA-GFP signals, the corresponding profiles were used for the systematic colocalization analyses. (B) Graphical illustration of the unbiased systematic ‘first harmonic’ analysis of deconvoluted 3d Hoechst signal inside representative cells in WT and Nal-treated strains. The Hoechst (blue) profiles indicate the signal intensities (integrated over the cell width) along the long cell axis at 9 in focus z heights with corresponding ‘first harmonics’ (dotted red curves, see Materials and Methods). Fluorescence signal distributions deviate significantly more from the first harmonics in WT compared to Nal-treated cells (Wilcoxon rank sum test, \( p < 10^{-41} \)), showing that Hoechst DNA stain distributions are perturbed in the latter. This analysis is independent of nucleoid length, which is altered in Nal-treated strains as compared to WT (S7A Fig.).
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References